

DIRECT AND INDIRECT TISSUE CULTURE MICROPROPAGATION  
AND GREENHOUSE PLANTLET EVALUATION OF SUPERIOR  
EUCALYPTUS GRANDIS HYBRIDS

By

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Abstract of Dissertation Presented to the Graduate School  
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**DIRECT AND INDIRECT TISSUE CULTURE MICROPROPAGATION  
AND GREENHOUSE PLANTLET EVALUATION OF SUPERIOR  
EUCALYPTUS GRANDIS HYBRIDS**

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A direct micropropagation system using three five-year-old superior *Eucalyptus grandis* hybrids was developed. Cultures were attempted from mature branches and from sprouts of girdled trees. Initiation of axillary buds was achieved with acidified MS plus 2.00 mg/l BAA and 0.5 mg/l NAF. High multiplying rate was obtained with 0.4 mg/l NAF. Shoot elongation was best on media with 2.5 mg/l of IBA and 2-1.5 mg/l of auxin. Serial subcultures in the multiplication media improved rooting significantly. Up to 90% rooting was achieved on 1/5 MS with 2 mg/l IBA. Rooted plantlets showed 95% survival after the first phase.

Tissue culture-derived plantlets were then compared to their half-sibs seedlings under natural greenhouse conditions. Plantlets and seedlings were sampled for a number of parameters and harvested every 45 days. Survival

was 66% and 68% for plantlets and seedlings, respectively. Seedlings had higher net photosynthesis, chlorophyll, and total nitrogen at early harvests, and the differences diminished with harvests. Foliar nitrogen was more correlated with net photosynthesis than chlorophyll. Plantlets had higher dry stem, leaf, and root weights and lower growth rates at all harvests; however, the difference decreased with harvests. Plantlets also showed higher below-ground to above-ground ratios. Plantlets and seedlings showed similar patterns with harvests for all the parameters studied. Significant differences were observed within plantlets and seedlings in dry weight accumulation and allocation, with similar patterns between plantlets and seedlings of the same parent.

Indirect micropropagation through axenic embryogenesis was attempted using immature inflorescences, floral parts, shoot tips, zygotic embryos and hypocotyle. Callus was induced in primary media with a combination of hormonal treatments of 2,4-D and kinetin and NAA and kinetin. No axenic embryos were observed when calli were transferred to secondary media with reduced hormonal concentrations. However, highly morphogenic callus was observed from hypocotyl explants at 2 mg/l of NAA and 1 mg/l of kinetin. Such callus produced a large number of plantlets through organogenesis.

## CHAPTER 1 GENERAL INTRODUCTION

Through vegetative propagation the unique traits of genotypes can be transmitted intact. Several techniques have been developed over the years, which include rooting cuttings, grafting, layering, and tissue culture. Tissue culture techniques (the aseptic growth of cells, tissues, and organs *in vitro*) have greatly increased the potential of asexual propagation by exploiting regenerative abilities of tissues more efficiently in a wider range of plants than is possible with conventional methods. Cell culture is also recognized as an important tool for genetic engineering, molecular, and physiological studies.

### History of Plant Tissue Culture

Totipotency, the ability of individual cells to develop into whole plants has forced the search for regeneration and propagation of selected genotypes through tissue culture. The totipotency theory was first proposed and clearly formulated by the German botanist Haberlandt in 1922 (Kusubige, 1974; Vasil and Vasil, 1982). Unfortunately all his attempts to regenerate plants from palisade mesophyll cells in a simple hormone-free media were not successful. But the importance of asepsis was

recognized from his experiments. Today, this theory has been verified through single cell culture (Nagai and Hildebrandt, 1980; Baskin-Jones and Baskin 1976).

In the 10 years following Hildebrandt's work very little progress was made. Various researchers reported unsuccessful attempts to culture cells and root tips in vitro (Street, 1977; Gauchat, 1980). By 1934 White reported positive results with tomato roots, where he obtained indefinite root growth by continual subculture.

Discovery of the auxin indole-3-acetic acid (IAA) in 1934 and its stimulatory effect on cell division opened a new era in tissue culture research. By 1936 the first indefinite growth of unorganized cells (callus) by adding IAA to the media was reported independently and simultaneously by White, Gauchat and Hildebrandt (Gauchat, 1980). Also White reported in the same year the differentiation of shoot from callus tissues of a tobacco hybrid (Gauchat, 1980). During the following years, while working with root culture, more attention was focused on the role of vitamins in plant growth (Street, 1977).

The major breakthrough in tissue culture came after the discovery of the cytokinin kinetin in 1955 (Miller et al. 1958). It was demonstrated that it could stimulate cell division. By adding cytokinins and auxins to the culture media plants could form callus tissues, roots, and/or shoots *de novo*. These studies ended in the now classical findings

of Skoog and Miller (1957). The basic regulatory mechanism underlying organogenesis involved a balance between the concentration and the ratio of cytokinin to auxin. A relatively high level of cytokinin to auxin favored shoot formation, while the reverse favored root formation. Intermediate ratios promote callus growth. They suggested that quantitative interaction between growth factors rather than specific morphogenic substances provided a mechanism for the regulation of the organogenic phenomena in plants. It was effectively the balance between the exogenous and endogenous factors interacting together which regulate organogenesis *in vitro* (Thorppe, 1981; Thorppe and Skoog, 1981). Tissue culture techniques have improved since then, and new developments have taken place such as suspension culture and somatic embryogenesis.

The impact of plant tissue culture on clonal propagation began with shoot-tip culture for virus free orchids by Horsch (1960). Today commercial growers are using shoot culture for clonal propagation of many plant species (Wareham, 1974; Vasil and Vasil, 1981).

### Plant Regeneration

Regeneration of plants via tissue culture can occur directly or indirectly. Direct regeneration is through proliferation of already existing organs to produce large numbers. This is sometimes termed shoot, bud, or root

culture. This system has been widely used in horticultural plants, mostly ornamental and fruit crops. Indirect regeneration, on the other hand, is the regeneration of whole plants through a callus phase. Callus can then differentiate into organs such as shoots and roots (organogenesis) or embryos (somatic embryogenesis).

Organogenesis and somatic embryogenesis are usually initiated with the formation of meristematic centers, with small densely cytoplasmic cells with large nuclei, in areas of intensive cell division activity, forming meristemoids (Thorpe, 1981). Such meristemoids are capable of giving rise to either shoots, roots or embryos. These adventitious organs which develop may arise directly from the original tissues or indirectly through callus phase.

Plant regeneration is a complex morphogenetic phenomenon controlled by intrinsic and extrinsic factors. Successful regeneration depends on: 1) selection of a suitable explant to start the culture, 2) proper choice of the nutrient media and 3) control of the culture environment.

### The Explant

The explant is one of the most important factors controlling the success of regeneration under culture conditions. The importance of the explant has been stressed by many researchers (Harsbidge, 1974; Thorpe, 1981). They

Identified many factors which influence the explant: genotype of the stock plant, the season of the year, the organs or tissues serving as the explant, the size and the physiological age of the explant, and the physiological state and age of the donor stock plant. Although totipotency is thought to be an inherent quality of plant cells, its expression is very much affected by such factors. Large genetic variability has been observed in terms of the readiness of explant response to tissue culture conditions. Also the more juvenile (physiologically young) the explants, the more they express their totipotency. The frequency of survival as well as the rate of development of explants in vitro may be proportional to the initial explant size. However, the rate of contamination is also proportional to the explant size. The ability of the explant to respond to tissue culture medium is also controlled by the culture physical environment. Those differentiation patterns that develop in culture depend on the genetic potential of the plant and the chemical and physical environmental conditions to which they are subjected.

### **Nutrient Media**

Composition of the nutrient media regulates to a large extent the development and differentiation pattern in culture. For successful culture media nutrients must be

available in both chemical composition and physical qualities. The major constituents of the media are inorganic salts, carbon source, vitamins, organic nitrogen, and growth regulators.

Actively growing tissues under culture require a continuous supply of inorganic salts. Of the macro-elements, nitrogen, potassium, phosphorus, calcium, magnesium and sulfur are usually required in larger quantities than other mineral nutrients (Bostery, 1949; Coles-Skine and Vasil, 1965). The micro-elements usually required in a very small amount are: iron, manganese, boron, zinc, molybdenum, copper, iodine, and cobalt (Coles-Skine and Vasil, 1965).

Sucrose and glucose are usually the standard carbon sources for tissue culture media (Coles-Skine and Vasil, 1965). Glucose has proven superior to sucrose only occasionally (Horschlag, 1974). Maltose and other disaccharides are generally found to be inferior to sucrose or glucose as the carbon source.

Although vitamins can normally be synthesized by plant tissues, their addition to culture media is also necessary. Lack of certain vitamins may limit growth and development in culture and therefore have to be incorporated in the media (Bostery et al., 1961). There is usually a requirement for thiamine (Coles-Skine and Vasil, 1965). Other vitamins usually added are nicotinic acid and

pyridoxine. Ascorbic acid has been reported by Murashige (1974) to prevent browning of explants. Common sources of organic nitrogen in tissue culture media include amino-acids such as glycine, glutamine and asparagine.

The combination of these nutrients (macro and micro-elements, carbon source, vitamins and organic nitrogen) in the media are usually referred to as basal media. Several basal media are now available (Murashige and Skoog, 1962; Gamborg et al., 1968; Gamborg, 1968; Weiss-Wein and Weill, 1968). But, Murashige and Skoog (MS) (1962) (Appendix 1) is by far the most used one. It is rich in nitrogen and other macro-elements, which tend to regulate the pH fluctuation.

The growth regulators auxins and cytokinins are the most important additives to the media, and they are considered responsible for stimulation and regulation of the morphogenic response. The most commonly used auxins are Indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA), and 2,4-dichlorophenoxyacetic acid (2,4-D). The widely used cytokinins are 6-benzyladenine (6-BA), 6-furfurylaminopurine (kinetin) and 8-(4-hydroxy-2-methyl-1-tetrahydropyrimidin-2-yl) adenosine (sestin). For most plants exogenous auxins are essential for *in vitro* cell division, and both auxins and cytokinins stimulate growth, development and differentiation. Other growth regulators are not usually used in culture media except in some systems especially with somatic embryogenesis.

Other media components include natural complements like coconut water, proteins, lipolipids, yeast extracts (Grisen-Adine and Yacil, 1983). They are usually used to enhance and improve responses. Growth media may either be in a liquid form or solidified with gelling agents, the most common of which is agar.

The pH of the media, which affects biochemical and physiological processes of plant cells, is another factor to be considered for successful culture. Values of 5.0 to 8.0 before autoclaving were suitable (Gamborg, 1983), and values as low as 4.2 were reported (Grisen-Adine and Yacil, 1983).

#### Eucalyptus Tissue Culture

Eucalypts are evergreen hardwood trees and shrubs of the genus Eucalyptus in the family Myrtaceae. There are over 400 species within this genus, ranging from straight single-stemmed trees up to 90 m in height to multiple-stemmed shrubby trees (McComb and Bennett, 1988). They are endemic to Australia and the islands to the north including Timor, New Guinea and Philippines. Because of their high productivity in short rotation plantations, they have attracted world attention. FAO (1984) estimated that some four million ha of eucalypts have been established worldwide.

Eucalyptus were first planted in Florida in 1898, but industrial plantations were not established until 1979 (Henry et al., 1980). Large plantations were established since then in south Florida through the cooperative efforts involving government, private land owners, and industry. Success of adapted *Eucalyptus* species in Florida may help meet the increasing demand for hardwood pulp in the U.S. Demand of *Eucalyptus* has been increasing recently with an annual rate of 18% (Blackwood et al., 1988). *Eucalyptus grandis* (Mill) gg. Salden was found through species trials to be the suitable *Eucalyptus* species for south Florida (Henry et al., 1982). The advantages of planting *E. grandis* include 1) an exceptional growth rate, especially compared to native hardwood species (Blackwood and Henry, 1981) and 2) an ability to coppice, making it an attractive species for short rotation biomass production.

An intensive breeding program was established in south Florida. Through four generations of selection, large genetic gain was realized in terms of biomass production. Parallel to that, vegetative propagation was considered for immediate genetic gain by capturing vigour of *E. grandis* hybrids. Vegetative propagation of eucalypts has been successful and large genetic gains have been realized in different parts of the world. Early trials were developed in Kenya and South Africa, where large genetic gain was reported (Gyasi and Gwala, 1987). The most successful

program was developed at Arcadia where millions of rooted cuttings were produced annually, which resulted in genetically uniform and better quality trees with higher biomass yield (Campbell and Ikemori, 1981, 1983). Brande (1984) estimated 113¢ gain from the cuttings in the Arcadia program. In Florida large numbers of rooted cuttings were produced in 1987, and their 15-month height and diameter measurements showed excellent growth (Rockwood et al., 1988). The success of vegetative propagation through rooted cuttings renewed and increased interest in *Eucalyptus* tissue culture.

*Eucalyptus* tissue culture initially concentrated on cell culture from young seedlings (Sensen, 1969; Jacquot, 1969; Radcliff and Stone, 1973). Use of nodal or microcuttings, without multiplication, was the subsequent objective and was achieved with *E. grandis* (Crawford and de Fouard, 1974; Crawford and Hibsch, 1978). Attention with many *Eucalyptus* species then shifted to multiplication of shoots before rooting (Huys et al., 1981; Hartney and Barker, 1980; Hartney, 1984; Darnell-Crawford et al., 1983). Since *Eucalyptus* tissue culture was extensively reviewed by Darnell-Crawford et al. (1982) and recently by Motomura and Bennett (1984), no major development was reported. One area, which is neglected in tissue culture studies of *Eucalyptus* and forest trees in general, is the post-culture performance of plantlets.

The major limitations of *Eucalyptus* tissue culture were reported to be sterility of field segment cuttings, limited number of shoots, difficulties of rooting shoots, and inability to induce embryos (somatic embryogenesis) or shoots (organogenesis) from callus.

### Objectives

A key element in the application of tissue culture to *Eucalyptus* species is the development of systems for regeneration of plantlets from cells, tissues, and organs. Development of such systems would result in large economic and immediate genetic gain for this important genus by capturing hybrid vigor. It will also open the door for silvicultural, physiological, and molecular studies. The main goals of this study were 1) to develop new direct and indirect tissue culture methods and techniques to overcome some of the limitations mentioned earlier for these genetically superior *E. grandis* hybrids and 2) to compare plantlets produced through direct micropropagation with their related half-sib seedlings. Accordingly this study was conducted and reported in three parts:

- 1) Direct micropropagation from apical-medial segments.
- 2) Indirect micropropagation through somatic embryogenesis.

- 2) Comparison of plantlets with their seed-mix samples in terms of dry matter accumulation and relative distribution, net photosynthesis, and chlorophyll and total nitrogen accumulation.

CHAPTER I  
DIRECT MICROPROPAGATION OF FIELD-TESTED SUPERIOR  
SUGARCANE GENOTYPE STRAINS

Introduction

Direct micropropagation is the most widely used system in tissue culture propagation industry (Vasil and Vasil, 1980; Lewis et al., 1984). This has led to more systematic studies of the components involved in direct micropropagation leading to a successful system. Murashige (1974), in one of the most thorough and read reviews of the subject matter, established a framework of standard terms which have often been referred to in subsequent literature (Thompson and Bland, 1981). He defined three stages which the cultures pass through as they develop into fully regenerated plantlets and discussed the requirements which should be considered for each stage.

Stages of Direct Micropropagation

Stage I is the establishment of an aseptic culture. The main objective of this step is simply to obtain uncontaminated cultures with a reasonable survival rate. This could be the main stumbling block especially for direct trees because of their long exposure to various microbial contaminants in the field.

Stage II is the multiplication of the shoots. The objective of this stage is to achieve rapid proliferation of shoots which can ultimately give rise to whole plants. The addition of various plant growth regulators and other nutrients to the medium can often control the growth and development of the cultured tissues. However, specific requirements seem to depend primarily on the species and the physiological age of the original explant.

Stage III is the rooting of the shoots produced in stage II and subsequent acclimatization of plantlets to the greenhouse and field conditions. The main objective of this step is production of full plantlets by inducing adventitious roots and then gradual hardening to normal greenhouse condition through mist stage.

Recently some modifications of these steps were proposed. Scharph and Mason (1981) working with ornamental plants suggested establishment of Stage "0" to prepare stock plants under hygienic conditions to produce healthier plant material and reduce the contamination problem at stage I. Rooting stage was suggested to be divided into three steps: elongation of shoots, rooting, and acclimatization. Elongation of shoots before rooting, which has received little attention in the past, is becoming more and more important and was suggested as a separate step (Fremwerst and Bouley, 1981; and Scharph and Mason, 1981). Also due to the difficulty of root initiation in many

species. Recent publications consider rooting as a separate stage (Janner and Oulida, 1981). The objective of the rooting step is to achieve the formation of functioning roots. The third step is the acclimation and hardening of plantlets to survive and grow under natural conditions.

### Considerations in Direct Micropropagation

Success in mass propagation of plantlets through direct micropropagation depends on the selection of a suitable explant and appropriate manipulation of the medium and the physical environment surrounding the culture for each stage of development. Wesseliq (1978) stressed the importance of selecting the right explant to achieve success, and several factors should be considered when making the selection. These factors include 1) the organ serving as explant, 2) the physiological or ontogenetic age of the organ, 3) the season in which the explant is obtained, 4) the size of the explant, and 5) the overall quality of the plant from which the explants are obtained.

The ontogenetic or physiological age of the cultured explant is a very important factor, particularly when dealing with forest trees. Most forest trees are selected for clonal purposes after they reach maturity and have been grown in the field for years. Generally, an inverse relationship exists between the age of a woody plant and the ease with which the tissue can respond to culture

conditions (Seng, 1961). Meristematic apices, the centers of growth and regeneration in plants, undergo changes when the plant matures; however, most trees have zones that retain a degree of juvenility longer than other areas of the tree. In the above-ground part of the tree, the least distance a shoot apical meristem is located from the base of the trunk, the more juvenile it generally is (Seng, 1961). The increased juvenility toward the lower and outer parts of the crown is explained in vegetative propagation of woody perennials. The most common practice is heavy and repeated pruning, or cutting down of the trees forcing sprouts or coppice or new shoots to develop from the lower parts of the trunk. These juvenile sprouts were laid down in the early life cycle of the tree and remained suppressed and juvenile until released. Such phenomena are described as arrested juvenility (Kunkel, 1977; Seng, 1961). Also the cambium near the base is more juvenile than the upper parts of the trunk and may retain some juvenile characteristics (Klassen, 1978). Similarly the coppice which arises *ex novo* from the cambium at the base of the tree is relatively juvenile. Another method for obtaining juvenility is aerial grafting of mature tissues and organs onto juvenile material (Seng, 1961).

Surface sterilizing to remove microbial contaminants is another important factor which should be considered during stage 1, and methods have been thoroughly described

by many authors [Marshalls, 1974]. Generally the tissues are placed into a disinfecting solution, such as sodium hypochlorite, and agitated for 15-20 minutes. In order to achieve surface contact between the disinfecting agent and the tissue, a few drops of a wetting agent are used. A compromise between the degree of sterilization and the viability of the explant has to be reached.

The nutrient medium used for each stage of culture must be considered carefully in term of its chemical and physical composition. The basal medium mostlly used is that devised by Marshall and Stone [1968] (Appendix I). Auxins and cytokinins are the most widely used growth regulators. The balance between cytokinins and auxins seems to be the most crucial factor for proper organogenesis and development. cytokinins are generally reported to aid in the proliferation of axillary shoots at the multiplication phase (stage II). According to Levin et al. [1988], cytokinins enhance shoot proliferation by inhibiting apical dominance. Addition of auxins and gibberellins also have been reported to enhance shoot proliferation [Martha et al., 1985]. The reduction of cytokinins and addition of auxins are often requirements for root development [Marshall, 1974]. Physical features that must be considered include physical qualities of the medium, light requirements, and temperature of the culture.

### First Direct Micropropagation of *E. grandis*

Early attempts at *Eucalyptus* micropropagation started in the early 1930s. Due to the problems of contamination and phenolic production in culture explants, most of the early research was done with aseptic young seedling explants. Organ culture of *E. grandis* was first reported with seedling explants (De Fossard et al., 1974; Greenwell and Nitsch, 1975), where they successfully rooted stem segments and transferred propagules to greenhouse conditions. Burtney (1980) and Burtney and Baker (1981) reported media for subcultivation and rooting for seedling nodal segments of 12 *Eucalyptus* species. Micropropagation of mature tissue of *E. grandis* was reported by Greenwell and Nitsch (1977), Parra and Greenwell (1980), Rao and Venkateswara (1985), and Lakshmi Devi (1986). Greenwell and Nitsch (1977) were able to control the phenol problems by washing nodal segments in distilled water for a few hours before rooting them directly. Parra and Greenwell (1980) used sprout explants and similar media as reported by Frensch and Boulay (1982) with some success. Rao and Venkateswara (1985) achieved shoot multiplication on a medium with very high BAP (over 10 mg/l) and NAA (10 mg/l) concentration. They found that rooting was the major problem. From these studies the most limiting factors for *Eucalyptus* direct micropropagation are production of a low number of shoots at the multiplication stage, abnormal

shoot growth at the elongation step, and low rooting response at the rooting step.

### Objectives

The main objectives of this study were to develop an efficient micropropagation cloning technique for three five-year-old field tested *E. grandis* hybrids and to overcome some of the propagation problems identified in the previous reports. At age 5.7 years these hybrids were reported by Mackinnon et al. (1987) to be superior in growth characteristics and frost resistance to 58 other clones. These 58 clones ( $30 \pm 1$ ) were selected as phenotypically superior trees in one of their growth characteristics in a base population and field studies after four generations of selection in the *E. grandis* breeding program in Florida.

This study tested the following hypotheses with these three *E. grandis* hybrids:

- 1) Sprouts survive as better explants than mature branches.
- 2) A high cytokinin to auxin ratio increases multiplication.
- 3) Graft transfer results in higher rates of multiplication.
- 4) Auxin and concentrations of auxin and cytokinin control elongation.

- b) Auxin coupled with reduced basal media improves rooting.
- c) Serial subculture in the multiplication medium increases the rooting ability of shoots.

### Materials and Methods

The methods followed in this study involved establishment of culture from nodal segments, followed by shoot multiplication, elongation, rooting, and acclimatization steps. The following procedures and conditions, unless mentioned otherwise, were shared in all the steps. All media, with hormones added, were adjusted to a pH of 5.4-5.5 and autoclaved for 15 minutes. The basal media of Murashige and Skoog (MS) (1962) with slight modification was used and will be referred to as MSB. The modification includes replacement of the amino acid glycine with 280 mg/l of glutamine, increasing thiamine to 5 mg/l, decreasing agar concentration to 5.0 g/l, and adding 10% coconut water to the basal media. Standard MS was used for the rooting experiments. Cultures were maintained in a growth chamber under 12 hr of light and dark with 16-24°C and 20-26°C temperature respectively. Fluorescent light at an average photon flux density of photosynthetically active radiation (PAR) of 10  $\mu\text{mol m}^{-2} \text{s}^{-1}$  was used. Culture containers were placed randomly in the growth chamber.

### Establishment of Callus (Stage 1)

Clones of three top *E. grandis* hybrids identified by Haskins et al. (1987) were used as source materials. Based on morphological criteria, Haskins et al. (1987) reported that clones 1 (1917) and 2 (2614) are hybrids of *E. grandis* x *E. canadensis* or *E. taraticornis* and clone 3 (1976) is *E. grandis* x *E. tomentos* and either *E. canadensis* or *E. taraticornis*. Sprouts and explants were induced by girdling five-year-old trees at one meter from the ground in February 1987. Node explants from mature branches and two-month-old sprouts were used as explants. Only healthy looking branches were selected. These were cut about 5 cm under the node into 10 cm long sections. Two leaves were left per section and each leaf was cut in half. These sections were washed with running water and surface sterilized by immersion in 70% ethanol for 2 minutes and then placed in 80% Chlorox which contained a small amount of detergent as a wetting agent for 15-20 minutes in sterile containers with constant agitation on a rotary shaker. Twenty-five sections per clone from sprouts and a similar number from mature branches were then rinsed with 4% Chlorox solution and cultured for five days in pre-initiation medium consisting of hormone-free MS. This step screened for contaminated explants and rid explants of phenols which were produced initially. The cultures were kept in the dark to reduce phenolic production. Explants

are known to produce phenolic compounds after wounding that could be detrimental to cultured explants (Kucera-Cresswell and Mitsch, 1977). After five days the uncontaminated explants were transferred to initiation medium, with each explant in a separate container. The initiation medium consisted of MSB supplemented with 0.5 mg/l of benzylamine purine (BAP) and 0.08 mg/l of naphthaleneacetic acid (NAA).

#### Shoot Multiplication (Stage II)

Various BAP and NAA combinations were tested with full strength MSB as a basal medium. Combination of BAP/NAA in mg/l were 0/0, 0.5/0, 1.0/0, 0.5/0.08, and 1.0/0.1. Fifteen shoot clumps per clone from the initiation step were assigned randomly to each treatment. Each individual clump was let on with 4-6 shoots. In addition, 15 single shoots per clone were cultured in 0.5 mg/l of BAP. Each clump or single shoot was cultured randomly into separate containers. Shoot multiplication was assessed after three weeks in culture by counting shoots approximately 2 cm or longer, which were clearly visible. After the first round of culture BAP at 0.5 mg/l was found to induce significantly higher numbers of shoots (Table 1) and was used routinely for further multiplication subculture. Clusters of shoots that formed were subdivided and subcultured in fresh multiplication media every three

media. In a follow-up experiment concentrations of shoot clumps on 0.4 and 0.8 mg/l of IAA was compared to 1.6 mg/l.

### Shoot Elongation Assays (IIa)

Preliminary studies with various hormonal concentrations of auxins, cytokinins and gibberellins with and without activated charcoal showed better results with combinations of high auxin and cytokinin. Thus led to testing elongation on treatments with combinations of high auxin (Indoleacetic acid (IAA)) and cytokinin (Kinetin) concentrations (Table I-1). Fifteen to be shoot clumps (SCS) are with approximately 35 countable shoots) per clone from the multiplication phase were then assigned randomly to each of the six elongation treatments. Each clump was cultured randomly into a separate container. The cultures were kept under dark conditions for two days and then transferred to 12 hrs of light. Elongation quality was assessed by counting the number of shoots longer than approximately 15 mm after four weeks in culture. Callus and root production were also evaluated at the same time. Further elongation subcultures were carried out in 1.6 mg/l IAA and 1.0 mg/l kinetin.

### Rooting and Elongation Assays (IIb)

Serial experiments determined the best nutrient and hormonal combination for rooting of elongated shoots (Table

10. Fifty to 60 single elongated shoots from the three clones were assigned randomly to separate culture tubes for each treatment. In the first and second experiments, different concentrations of MB ( $1/4$ ,  $1/3$ , and full strength) plus hormone treatments were compared. In order to further detail the mineral requirements, a third experiment was conducted using 2 mg/l of IBA with boron, calcium, and boron and calcium. Boron and calcium were added in concentrations similar to that in  $1/4$  MB as 1.2 mg/l of boric acid ( $H_3BO_3$ ) and 11.6 mg/l of calcium chloride ( $CaCl_2$ ). A range of pH (4.0, 4.5, 5.0, and 5.0), with the best hormone and basal nutrient combinations identified in the previous experiments, was also tested. Variability between the three clones in terms of their rooting response was also studied by observing the rooting response of 100 to 140 elongated single shoots per clone on 2 mg/l of IBA and  $1/4$  MB. In all of these experiments the cultures were kept under dark conditions for the first five days (after Barnes-Cresswell et al. 1982) at 20-24°C, and then transferred to 12 hr of day and night. Rooting was evaluated after 17 days in culture, when after root tips started to emerge. Rooting responses on 2 mg/l of IBA and  $1/4$  MB from the serial rooting experiments separated by 1-2 subcultures on the multiphasic media were compared (Table 2-5).

Protophytes were removed from culture tubes with a minimal amount of agar without damaging the roots. Roots of each protophyte were then washed in a solution of fungicide (benlate) to remove remaining agar and prevent fungal contamination. A total of 112-128 protophytes per clone were then planted in trays containing a commercial potting mixture known as Metrosia mixture 100, which consists of peat, vermiculite, vermiculite, sphagnum, sand, and processed bark. They were then placed under mist for three weeks after which survival was assessed. They were then potted in tubes and transferred to natural greenhouse conditions.

### Statistical Analysis

Statistical Analysis System (SAS, 1985) was used for the statistical analysis for the elongation and multiplication data (Appendix 1). Hormonal treatments and clones were considered as fixed effects in the analysis of variance procedures. Chi square test was used for rooting and survival data analysis.

## Results

### Establishment of Culture (Stage 1)

Similar to other reports for field grown forest trees, contamination was a major problem with explants from mature branches. After five days in the pre-initiation

media, 100% contamination of the culture material terminated this portion of the study. Contamination was only 8% with spread material on the pre-initiation media after the first nine days. Contamination increased to 40% for the sprout-explants after two weeks in the initiation media. The initiation media used in this study produced 3-12 shoots per surviving explant in six weeks.

### Shoot Multiplication Stage III

Highly significant variation was observed between the treatments in the first experiment, while the interactions between the multiplication treatments and clones, and the main effect of clones were not significant in terms of number of shoots produced at the multiplication stage (Appendix E). All the hormone combinations produced significantly more shoots than the hormone-free media (control) (Table 2-1). Massive shoot proliferation was observed with 0.4 mg/l of BAP and clamp transfer producing 150 shoots 1 cm or longer per original clamp (Figure 2-1). Increasing BAP concentration to 1.6 mg/l reduced the number of shoots by 40% compared to 0.4 mg/l of BAP, and evidenced yellowing of leaves. Addition of KIN did not increase the multiplication rate; instead elongation of existing shoots occurred with a lower rate of multiplication compared to 0.4 mg/l of BAP alone. Further studies with 0.4 and 0.8 mg/l of BAP resulted in insignificant reduction in the

Table 2-1. Mean number of countable shoots (3 mm or larger) produced per clump and single shoot transfer on multiplication media with various MAA and MAF combinations.

| Exp. | MAA  | MAF  | Type of shoot      | Average number of shoots |
|------|------|------|--------------------|--------------------------|
| 1    | mg/L | mg/L | Identify           |                          |
|      | 0.0  | 0.0  | clump <sup>1</sup> | 175 <sup>a</sup>         |
|      | 0.0  | 1.0  | clump              | 82 <sup>a</sup>          |
|      | 0.05 | 0.0  | clump              | 48 <sup>c</sup>          |
|      | 0.1  | 1.0  | clump              | 88 <sup>a</sup>          |
|      | 0.0  | 0.0  | clump              | 17 <sup>b</sup>          |
|      | 0.0  | 0.0  | single             | 14 <sup>a</sup>          |
| 2    | 0.0  | 0.0  | clump              | 133 <sup>a</sup>         |
|      | 0.0  | 0.0  | clump              | 134 <sup>a</sup>         |
|      | 0.0  | 0.0  | clump              | 133 <sup>a</sup>         |

<sup>1</sup> Indicates significant variation at 5% level. Means with the same letters in the same experiment are not significantly different according to Duncan's multiple range test.

<sup>2</sup>Clump refers to 3rd or older with 3-5 shoots at the time of transfer to the multiplication treatments.

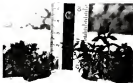


Figure 2-1. *Baculypion grandis* shoot multiplication on 0.1 mM BSA (left) and elongation on 2.0 mg/l BSA and 1 mg/l auxin (right).

shoot multiplication rate compared to 2.5 mg/l (Table 2-8). Transfer of small shoot clumps was better than the single shoot transfers with 10 times more shoots.

#### Shoot Elongation (Stage IIIA)

Elongation, similar to multiplication, was affected by the elongation treatments, with highly significant variation between them. Interaction between clones and the treatments or the main effect of clones was not significant (Appendix 5). Elongation of shoots was best on 2.5 mg/l of IBA and 1-2.5 mg/l of auxin (Table 2-9). This combination increased the length of the shoots to 15-25 cm in 4 weeks and expanded the leaves similar to normal seedlings of eucalypts (Figure 2-1). Transfer of 50 countable shoots from multiplication media resulted in 17 elongated shoots. The auxin and cytokinin balance was critical in triggering different morphogenic responses (Table 2-9). Reduction of auxin to 0.5 mg/l produced significantly more callus; however, further reduction to 0.2 mg/l inhibited significantly new roots in addition to callus formation.

#### Rooting and Hardening (Stage IIIB)

Rooting of elongated shoots depended on the basal media and the kind and concentrations of the hormones applied (Table 2-10). Reducing BA concentration to 1/4 increased rooting quality and reduced callus production,

Table 2-3. Mean number of elicited shouts > 10 ms per sleep (50-55 counts/s) from the multiplication stage per sleep on various combinations of LNA and acetic and observed calls and roosting responses.

| LNA<br>mg/L | Acetic<br>mg/L | Elicited shouts  | Calls           | Roosts          |
|-------------|----------------|------------------|-----------------|-----------------|
| 3.0         | 0.0            | 4 <sup>abc</sup> | 3 <sup>ab</sup> | 3 <sup>ab</sup> |
| 3.0         | 0.3            | 0 <sup>b</sup>   | 4               | 1               |
| 3.0         | 3.0            | 27 <sup>ab</sup> | 1               | 0               |
| 3.0         | 3.3            | 27 <sup>ab</sup> | 1               | 0               |
| 4.0         | 0.0            | 11 <sup>b</sup>  | 3               | 1               |
| 4.0         | 3.0            | 20 <sup>ab</sup> | 1               | 0               |
| 6.0         | 0.0            | 0 <sup>a</sup>   | 0               | 0               |

\* Indicates significant differences at 5% level between treatments.

<sup>1</sup> Means with the same letters are not significantly different at 5% level according to Duncan's multiple range test.

<sup>2</sup> Quantitative scale of 1-5 with 1 means less, "0" indicates not observed.

Table 2-1. Rooting response and callus production of single elongated shoots after 17 days in culture of three serial experiments (Exp.) with different nutrient and hormonal concentrations.

| Exp.<br># | IBA<br>mg/l | BAP<br>mg/l | Serial media                                   | Rooting<br>%      | callus <sup>b</sup> |
|-----------|-------------|-------------|------------------------------------------------|-------------------|---------------------|
| 1         | 0           | 0           | M0                                             | 66.7 <sup>a</sup> | 0                   |
|           | 1.0         | 0           | M0                                             | 79.5              | 0                   |
|           | 1           | 0           | M0                                             | 73.8              | 0                   |
|           | 1.0         | 0.5         | M0                                             | 0                 | 0                   |
|           | 1.0         | 0.5         | M0                                             | 0                 | 0                   |
|           | 0           | 0           | 1/3 M0                                         | 64.0              | 0                   |
|           | 0           | 0           | 1/4 M0                                         | 66.4              | 0                   |
|           | 0           | 0           | 1/4 M0                                         | 76.0 <sup>a</sup> | 0 <sup>c</sup>      |
| 2         | 0           | 0           | 1/4 M0                                         | 68.3              | 0                   |
|           | 0           | 0           | 1/4 M0                                         | 66.1              | 0                   |
|           | 0           | 0           | 1/4 M0                                         | 70.0              | 0                   |
|           | 0           | 0           | 1/4 M0                                         | 70.0 <sup>a</sup> | 0 <sup>c</sup>      |
| 3         | 0           | 0           | 1/4 M0                                         | 66.0 <sup>a</sup> | 0 <sup>c</sup>      |
|           | 0           | 0           | M0 mineral added <sup>a</sup>                  | 66.0              | 0                   |
|           | 0           | 0           | 1.0 mg/l BAP00                                 | 66.0              | 0                   |
|           | 0           | 0           | 110 mg/l CaCl <sub>2</sub> .2H <sub>2</sub> O  | 66.0              | 0                   |
|           | 0           | 0           | 1.0 mg/l BAP00                                 | 66.0              | 0                   |
|           | 0           | 0           | +110 mg/l CaCl <sub>2</sub> .2H <sub>2</sub> O | 66.0              | 0                   |

<sup>a</sup> Significant variation at the 0.5 % level between treatments within each rooting experiment.

<sup>b</sup> IBA was used with distilled water, no mineral nutrient were added.

<sup>c</sup> Qualitative scale of 1-4 with 1 means less callus and "0" not observed.

however provision of only boron and sodium resulted in poor rooting percentages and quality. Sulfonamide IBA at more than 1 mg/l resulted in increased callus and reduced rooting quality. Addition of BAP, at low concentration, inhibited rooting. A pH in the range of 4-6 was found suitable with 100-100% rooting, but pH of 5-5.5 gave best rooting quality. There was no significant variation between the three classes for the number of shoots which rooted (Table 3-4). However, there was significant variation in the number of roots per shoot. Class 1 showed higher percentage of shoots with more than 15 roots per shoot (Table 3-4). Serial subculture on the multiplication medium increased rooting percentages significantly on 1/4 MS and 1 mg/l of IBA (Table 3-4).

Rooted propagules were successfully hardened in a mist-greenhouse with survival of 80% across the three classes, with significant clonal variation (Table 3-4). Class 1 survived poorly compared to the other two classes. Survival was 100% on further transfer to normal greenhouse conditions.

## Discussion

### Establishment and Multiplication

Mass vegetative propagation of hybrids is one of the main objectives of micropropagation through tissue culture.

Table 3-4. Rooting of detached shoots of three clones on 2 mg/l of IBA and 1/4 MS after 17 days in culture and survival in the mist-growthhouse after 3 weeks.

| Clone    | Number<br>of shoots | Rooting |                   | Survival            |                 |
|----------|---------------------|---------|-------------------|---------------------|-----------------|
|          |                     | rooting | with +15<br>roots | Number<br>of plants | Survival        |
|          |                     | k       | k                 |                     | k               |
| 1 (2037) | 140                 | 57.8    | 34.0 <sup>a</sup> | 136                 | 50 <sup>a</sup> |
| 2 (2038) | 138                 | 58.6    | 35.4              | 135                 | 57              |
| 3 (2159) | 138                 | 57.8    | 46.7              | 138                 | 57              |
| Average  |                     | 57.8    | 38.8              |                     | 52              |

<sup>a</sup> Chi square test showed significant variation between the three clones at 5% level.

Table 1-3: Root rooting response of the three clones on 2 mg/l of IBA and 1/4 MS after serial subculture on multiplication medium.

| Subculture number | Number of shoots | Rooting %         |
|-------------------|------------------|-------------------|
| 1                 | 80               | 88.4 <sup>a</sup> |
| 2                 | 80               | 90.8              |
| 3                 | 80               | 90.0              |
| 4                 | 400              | 93.8              |

<sup>a</sup> Chi square test showed significant variation at 5 % level.

It is especially important for forest tree species to increase productivity by capturing hybrid vigor.

The major critical factor of propagation of questionably "pure" trees is the difficulty of culturing mature tissues and sterilizing them after exposure to field conditions for long periods. However the genus *Eucalyptus* has the ability to coppice and sprout (Sawney 1983), thus producing juvenile material less exposed to sporogametes. In this study contamination of sprout-regenera was 48%, while those from mature branches were 100% undamaged and therefore amenable. More stringent sterilization conditions resulted in Browning and death of tissues, as was reported by Bennett and Hootsch (1983). This may indicate the importance of stage "B", as proposed by Delcamp and Burns (1983), where induction of new sprouts by girdling or felling trees will provide plant material less exposed to contamination.

The hormone-free pre-initiation medium was found to be useful in reducing phenolic production and its detrimental effects. It also served as a suitable medium to screen for contamination. The shoot initiation media, similar to that reported by Frenchet and Boulay (1982) and used by Farris and Greenwell (1985) gave satisfactory results, however it took 4-7 weeks to produce 10-12 shoots. Conceivably the use of multiplication media directly will speed the propagation process. Unavailability of large

numbers of sterile explants restricted experimentation with different combinations of hormones at the initiation stage.

As was hypothesized, cytokinin proved to be the essential growth regulator for high rate of shoot multiplication along with clump transfer. Multiplication was best on 8.4 mg/l of BAP and 600 mg/l with the three clones, similar to that for *L. camaldulensis* seedlings (Warwick et al., 1983). Unlike other reports with *L. grandis* (Greenwell and Ribeth, 1978; Purne and Greenwell, 1983; Rao and Venkateswara, 1985, and Lakshmi Sita, 1986), no need was found to add auxin except what may be present in coconut water (Rao and van Staden, 1984). Cluster transfer was observed to be better, and this has practical implication since it is easier and more economical to transfer shoot clumps rather than isolating single shoots.

### Shoot Elongation

The elongation medium reported in this study could have significant practical application for eucalyptus micropropagation and other species. It is the first time to my knowledge that high auxin concentrations were used for elongation. The main objective of this critical step, which was mostly ignored in eucalyptus tissue culture, is to produce robust (long and strong) shoots for efficient rooting and further survival. Quairns et al. (1974) reported that a period of elongation following

multiplication predisposes the shoots to root in response to rooting treatments. Use of gibberellins was tried by Frenschel and Bailey (1983) with *G. grandis*, where they suggest the use of 2.1 mg/l of gibberellic acid with activated charcoal. Higher concentrations of gibberellins tended to produce stronger leaves and stems. In this study, it was found, similar to the hypothesis, that the ratio and concentration of auxin and cytokinin can influence rooting. IBA and NAA at 2.5 mg/l and 1-2.5 mg/l, respectively were optimal for production of strong elongated shoots in 10% of the countable shoots transferred from the multiplication phase. Auxins were suggested to act directly in the induction of cytodifferentiation, elongation and cell division (Radaba and Kozminski, 1988). Another possible advantage of using high auxin at the elongation stage is the improvement of subsequent rooting. Motesh and Bennett (1988) stressed the carry-over effect from multiplication of elongation media on subsequent rooting ability. They suggested that use of gibberellins for elongation seems to greatly reduce rooting. Also omission of the auxin NAA from the multiplication media reduced subsequent rooting by 24%. This may explain the high rooting percentages (up to 80%) obtained in the present study.

### Rooting and Hardening

In agreement with the hypothesis, rooting was best in reduced basal nutrient media with IAA, and it improved with subculture in the multiplication media. Rooting of forest tree shoots is one of the most critical factors in the success of the direct micropropagation system. It is the only step in which organogenesis and de novo formation of an adventitious organ occurs. Rate of adventitious root formation generally declines with increasing age of the explant (Kasper and Gorman, 1987). Rooting of different microphytes was identified as the most limiting factor by Gupta (1981) and Rao and Venkateswaru (1988). This is why in this study considerable emphasis was given to this step in order to define the hormonal, nutritional, and pH requirements of rooting. Rooting was best in a medium with only auxin and reduced basal nutrients concentration. The combination of 2 mg/l of IAA and 1/4 MS with a pH in the range of 3-5.5 gave the best results. The best concentration of IAA was double that previously recommended for microphytes, where 1 mg/l of IAA was suggested (Hartney, 1988; Frenkel and Sealey, 1982; and Mohsen and Bennett, 1988). Increasing IAA to more than 2 mg/l tended to produce more callus. Addition of BAP at 0.2 mg/l inhibited rooting completely in the first 17 days.

Since rooting was improved with the reduction of MS from full to 1/4, experiment 2 was conducted to test

Kilgus's [1978] suggestion that only boron and calcium are the necessary minerals for rooting of shoots of woody plants stands. Kilgus [1978] argued that applied nutrients may not be needed during the rooting stage because nutrients are passively transported from the shoots, except calcium and boron where they are poorly transported in the phloem. However, in this study inclusion of only boron, calcium, and the combination of boron and calcium resulted in poor rooting, even less than with no minerals. This may indicate that nutrient stress to a certain extent can improve rooting and additional stress reduces it.

Continued subculturing in the multiplication media improved the rooting response significantly (Table 2-8). Similar results were obtained by Gupta et al. (1981) working with *E. glaucoloma*. Such a phenomenon was considered to be a sign of rejuvenation (Gupta, 1981).

Transfer of propagules was achieved with high survival, and propagules have successfully survived greenhouse conditions. Although class 2 had significantly more roots per shoot at transfer time (Table 2-8), a significantly lower survival percentage at the elite-greenhouse was observed (Table 2-9). This may indicate, similar to the recommendation by Bernard-Cousens et al. (1982), that transfer from rooting media should be done as soon as a few roots are produced. In this regard rooting

variability between clones should be considered in the transfer to the field conditions.

### General Discussion

Forcing relatively juvenile sprouts from mature trees by girdling (Kemp, 1932) and continued subculture was the key for successful micropropagation of the *E. grandis* clones used in this study. It allowed for a high multiplication rate, and high rooting percentage. Kemp (1932) discussed the retention of juvenility in some parts of mature trees. He mentioned that in the above ground part of the tree, juvenility, in general, increased towards the base of the trunk and away from the apex of the terminal stem.

Using the procedure reported in this study, more than a million propagules ready for operational planting can be produced from a single sterile explant within a year (Figure 3-8). This can be achieved by division of shoots into clumps and continued subculturing on the multiplication medium. Subsequent elongation of the shoots and continued recycling of unelongated shoots can recover all of shoots produced at the multiplication phase. Rooting and survival in the greenhouse and the field could be achieved with high rate of success. With such a high rate it could be an integral part of breeding program for production of genetically uniform plantlets.



Figure 3-2. A scheme for direct micropropagation for *Eucalyptus grandis* from sprouts or coppice rooted segments.

The greatest operational cost of propagule production is labor. No reliable figures are available, but it is estimated to account for 40-50% of the total cost. Franclet and Bouley (1982) estimated the cost of eucalypt propagules to three times that of seedlings. Most of the cost consists of manual cutting and transferring of cultured materials. Automation of some or all parts of the system could greatly reduce the cost in this process. Because of clump transfer, and the uniformity and even age of shoots in this study at the multiplication and elongation phases, this may be an excellent system for automation. A microprocessor as was described by Levin et al. (1988) can be used for separation, sizing, and distribution to culture vessels. Another area of cost reduction could be through by-passing the 10 days rooting step. The elongation medium developed in this study can produce shoots longer than 10 mm, which can be sliced and rooted under high condition directly.

#### Conclusions and Recommendations

Direct micropropagation of *E. grandis* hybrids was shown to produce a large number of plantlets, at least three times more than was reported with *E. grandis* and other eucalypts. However, since many chemical, physical, and physiological factors can affect each stage of a plantlet development, no claim is made that the system

developed here is the best. More research and investigation are needed especially to reduce the cost. Direct rooting under mist conditions is one area where substantial reduction could be obtained. Another area of interest is the role of hormones, especially auxin, in cylogenesis and production of stronger stems at the elongation stage.

Although this study was conducted with three genetic lines (hybrids), no significant variation between them was observed. This may suggest that the system developed in this study can be used, at least in a broader sense, for *E. grandis* and its hybrids. The following steps for plantlets production are recommended:

- a) Find a good candidate to induce sprouts and supply at the right time of the year, which is January to February in Florida.
- b) Collect nodal segments from 1-2 month-old sprouts.
- c) Establish culture on hormone-free media after sterilization for five days (3-7). Be sure to wash the explants thoroughly. Leave cultures in dark to reduce phenol production.
- d) Transfer unsterilized segments to initiation medium with concentrations around 0.5 and 0.05 mg/l of BAP and IAA, respectively. Cut segments lower end, which were exposed to sterilization agents, and check regularly for contamination.

- 1) Transfer shoots produced at the initiation phase as small clumps (4-6 shoots per clump) to multiplication medium. Use MSB with a range of 2.4-3.6 mg/l of IAA with 12 hr photoperiod at this stage and temperature of 25° C. Subculture shoot clumps every 3-4 weeks.
- 2) Transfer shoot clumps (10-20) to elongation medium, with 2.5 and 1.2-1.5 mg/l of IAA and GA3, respectively.
- 3) Remove shoots longer than 15 cm and place them vertically into rooting medium, with IAA around 2 mg/l of IAA and 1/4 MS. Leave culture in dark for 3-5 days and then to 12 hr of photoperiod.
- 4) Leave shoots in the rooting media until a few root tips emerge, usually between 15-20 days.
- 5) Remove plantlets from the rooting media and wash agar from the roots and dip them briefly in fungicide solution to avoid contamination.
- 6) Transfer propagules to mist conditions for 2 weeks and then to normal greenhouse with frequent watering.

These recommendations can be used as guidelines for developing systems which might suit different conditions, or as a starting point for new research leading to a more efficient and better system.

Further research is needed mostly to reduce the cost. Development of ways and means to automate the system will cut the cost. Direct rooting of elongated shoots under high conditions would eliminate the in vitro rooting steps.

Research could also be directed to understand the hormonal role in the elongation and tapering of axian clonets. The rejuvenation observed with continued subculturing in the multiplication step also deserves more attention.

CHAPTER 3  
COMPARATIVE GREENHOUSE STUDY OF EUCALYPTUS SEEDLING  
PLANTULE AND HALF-SIEVE SEEDLING.

Background

Large scale micropropagation of selected eucalypts can greatly increase and hasten genetic gains for tree improvement programs. Seedling seed orchards rely on additive genetic effects while mass cloning can capture the entire genetic potential of selected trees. Efficient tissue culture propagation of elite trees for operational planting would eliminate time required for seed orchards to be established and reach seed bearing age. As described in the previous chapter, micropropagation of *E. grandis* was successful in producing large numbers of plantlets. The next step is to determine how plantlets adjust to post-culture conditions, once past the vial stage.

These culture-derived plantlets originate from adventitious shoots and roots. Also while under culture conditions they are exposed to exogenous factors such as growth regulators and nutritional treatments for establishing the culture, multiplying, elongating, and rooting of shoots. These exogenous controls of development may not be as precise as zygotic embryo development in a normal seed. These factors may lead to temporal or

parental physiological changes, which, in turn, can change the growth pattern in the plantlets. Parental physiological (epigenetic) change can reduce the genetic gain meant to be captured by tissue culture-propagation.

Operational planting of vegetatively propagated eucalypts rooted cuttings has been very successful (Campbell and Tharwat, 1985 and 1986; Brande 1986, Medina and Garcia, 1987; Lockwood et al., 1988). However, for plantlets, because of the above mentioned reasons, a great deal of information about their performance at the nursery (or greenhouse) and the field is needed. Only three studies with eucalypts have compared height and stem volume of plantlets and seedlings. Bennett et al. (1988) have reported differences between *Eucalyptus marginata* (Caven) seedling and plantlet heights over two years. Also, Kucuradze et al. (1988) and Kump et al. (1987) compared stem growth of *E. citriodora* and *E. teretifolia* plantlets with seedlings over a period of 18 months and reported significant differences. This may indicate different growth patterns between seedlings and plantlets.

Vegetative propagation through tissue culture is usually used as a tool to capture genetic potential of elite trees for higher productivity. Thus there is a need to quantify the productivity of plantlets in terms of their survival, net photosynthetic efficiency, accumulation of dry matter in root and shoots, and nutrient accumulation.

### 3.1.1. Photosynthesis

Photosynthesis in green plant cells is one of the most important physiological processes and we indicate the effect of micropropagation as compared with plants grown from seeds. It is a process which utilizes light energy to produce carbohydrates from carbon dioxide ( $\text{CO}_2$ ) and water. Photosynthesis or carbon fixation can be viewed as a series of diffusion resistances at the stomata and the chloroplast level (Teng, 1982), and tissue culture conditions can affect these resistances. Numerous reports have shown that plant cells under tissue culture conditions have reduced amount of chlorophyll and photosynthesis. These effects have been attributed to the special growth conditions in vitro such as sugar addition to the culture media, hormonal treatment, and physical conditions around the culture. Exogenous supply of sugar (photoheterotrophic or semi-photoheterotrophic nutrition mode) was observed to reduce chlorophyll and photosynthesis on plant cell cultures by Davey et al. (1971) and others (Kawano 1978). Reduction of sugar supplies was accompanied by increase in chlorophyll and photosynthesis. Exogenous phytohormones, which are essential for growth and development, also have significant effects on chlorophyll development and photosynthesis. Auxins tend to inhibit chlorophyll development (Davey et al. 1971) while cytokinins was essential for chlorophyll synthesis in tobacco call culture

(Reynolds et al., 1968). Chlorophyll formation is inversely proportional to enhanced growth, whether it be due to hormonal or sugar treatments (Mansmann 1964). Light quality and quantity were also reported to affect specific amino, protein synthesis and ribulose biphosphate carboxylase synthesis (BBS1500) (Friedlander and Richter 1962, and Mansmann 1968).

The question of whether these changes which take place during culture are permanent or temporary and will revert to normal during the post-culture phase (under complete photoautotrophic conditions) has been addressed by a limited number of studies. Gross and Aston (1974) related the major problems commonly associated with cauliflower plantlets to their insufficient photosynthetic capability and inability to regulate transpiration. Cultured plantlets of *Brassica glauca* had lower chlorophyll levels and  $\text{CO}_2$  fixation rates than non-cultured plants (Gross and Aston 1974). Carbon dioxide fixation of strawberry leaves was found insufficient to sustain autotrophic growth; however it increased significantly in the newly formed leaves (Gross and Wilkin, 1965). Red raspberry plantlets exhibited significantly lower net  $\text{CO}_2$  uptake rates than field control plants (Donnelly and Tidmore 1964). Chloroplast ultrastructural differences were also observed between sweetpotato plantlets and cuttings (Lee et al. 1980). They also reported lower growth vigor for plantlets.

### Dry Matter Accumulation and Allocation

Photosynthate allocation could also be affected by culture conditions. The balance maintained by plants between the growth of their various organs was considered as an indicator to certain environmental, physiological and genetic causes (Shepherd and Leclercq, 1964). Allocation of photosynthate to dry matter in the root and shoot sinks is governed by interrelated physiological needs and dependencies. Roots (1977) indicated presence of a functional equilibrium of shoot growth and root growth which depends on the flow of nutrients from the roots to top and photosynthate to roots. Baskin (1968) and Thornley (1973) mentioned that photosynthate allocation seems to be partitioned in an inverse relation proportional to relative activity. Allocation is also conditioned by pretreatments, genetic makeup, and environmental conditions.

In this regard the shoot:root ratio serves as an index for growth pattern. Shoot:root ratio for a given species was reported by Roots (1977) to have characteristic changes with physiological aging and development. This would also indicate the possibility of differences between plantlet and seedling growth patterns. Endogenous growth regulators also play a central role in the growth pattern of plants. Tissue culture conditions can alter the balance between endogenous hormones by the addition of exogenous hormones.

In addition, physiological age of plantlets is different than seedlings. Thus, in turn, can cause difference in the shoot:root ratio between plantlets and seedlings.

The growth pattern of loblolly pine plantlets was reported to be different from that of seedlings under similar conditions (Jesch 1978, McIsaac and Winkiewicz 1981). Early growth of plantlets (the first 3-5 weeks) was very slow compared to seedlings of similar age (McIsaac and Winkiewicz, 1981). They also found that only after 4-5 weeks did growth of plantlets become rapid, but still slower than seedlings. McIsaac and Allen (1984) compared plantlets of loblolly pine to seedlings to quantify growth and developmental differences. Over a period of 18 weeks in the greenhouse, seedlings grew to a larger size than the tissue culture plantlets. However the relative growth rates were approximately equal. *Larix laricina* plantlets were reported to be shorter and have more branches in the greenhouse and were taller in the field as compared to seedlings (Pettit et al. 1984). However Manurestian et al. (1982) reported that more above ground biomass of plantlets after 12 months in the field but only 54% after 18 months in the field as compared to seedlings.

### Nitrogen Accumulation

Efficiency in nitrogen uptake is another important aspect which needs to be considered. Nitrogen uptake and

utilization efficiency in biomass production requires that processes associated with absorption, translocation, and redistribution of nitrogen operate effectively (Mull et al., 1984). Tissue culture conditions and the physiological age difference of plantlets, as compared to seedlings, have the potential to alter these processes and result in different patterns. McLeod and Allen (1984) indicated that the major difference between labially pine seedlings and plantlets was their nutrient uptake, as seedlings had significantly higher nitrogen and potassium uptake. However plantlets were more efficient in nutrient utilization.

### Objectives

It is possible that tissue culture conditions may influence *E. grandis* plantlet productivity during and after culture phases. The goal of this study was to test the hypothesis that plantlet productivity and growth pattern from three top *E. grandis* clones is different from their seedling half-sibs under greenhouse conditions similar to those used to raise seedlings for commercial planting.

Specific objectives of this study were to test the following hypotheses:

- 1) Net photosynthesis, chlorophyll content and foliar nitrogen content of plantlets are different from their respective half-sib seedlings.

- 3) Survival, growth, and growth pattern of plantlets are different from their respective half-sib seedlings.
- 4) Nitrogen accumulation in leaf, stem, and root of plantlets is different from their respective half-sib seedlings.
- 5) Plantlets from each genetic line will have a similar pattern to their respective half-sib seedlings.

#### Materials and Methods

##### Propagation and Germination

Plantlets were propagated as described in chapter 2 from clones 2817, 2814, and 2728 in study ORCH-37 at Palmdale, Florida. seeds were collected in March 27 from the same mother-trees (situated in ORCH-37 [E. grandis seedling seed orchard near Lantana, Florida]). Seeds were germinated and plantlets were hardened under mist conditions concurrently from March 7 to March 21, 1988. The experiment started after both the germinated seedlings and hardened plantlets were transplanted to larger size pots and could withstand greenhouse conditions. The experiment started on April 1, 1988, when most of the seedlings had their first two leaves (after the cotyledonary leaves) and the plantlets had 4-5 leaves. They were transplanted to pots filled with 1:1 sand and Metrosmix 508, which is a

commercial potting mixture consist of peat, vermiculite, setting agent, weed, and processed bark. Plantlets and seedlings were fertilized twice a week with full strength Hoagland nutrient solution until September 3, 1968.

### Design

Baseline data collected at the start of the experiment on April 1, 1968 (harvest 1), included stem, leaves, shoot, root, and whole plant dry weight, and foliage, stem, and root total nitrogen percent. Four blocks were established in the greenhouse and each block was divided into four plots (Appendix 1-1). Seedlings (8-12 per family) and plantlets (4-8 per clone) were randomly assigned within each plot. Stomata, photosynthesis, chlorophyll and nitrogen measurements were collected four times at 44 days intervals following harvest 1: harvest 2 (May 9, 1968), harvest 3 (June 18, 1968), harvest 4 (July 28, 1968), and harvest 5 (September 3, 1968).

### Net Photosynthesis

A system similar to Williams and Kemp (1978) was used to measure net photosynthesis. A steady-state gas exchange conducted with a differential infrared gas analyzer (an Analytical Development Company carbon dioxide analyzer (885 KE 10) in an open system. A cuvette (13" high and 4" in diameter) equipped with several devices to control

temperature, air circulation, and relative humidity was connected to the infrared gas analyzer. Temperature inside the cuvette was kept near 27° C using a transparent plastic water bath that surrounded the sides of the cuvette. Relative humidity of the air entering the cuvette was adjusted to 80%. A fan was housed on the side of the cuvette for adequate mixing of air inside the cuvette. A high intensity sodium vapor-pressure lamp was installed over the cuvette and separated from the cuvette by a heat absorbing water bath. Average photon flux density of photosynthetically active radiation (PAR) was 1800-2000  $\mu\text{mole m}^{-2} \text{s}^{-1}$  at the cuvette level. Net photosynthesis stabilized after 15-20 minutes after enclosing the plant inside the cuvette, at which time net photosynthesis was measured.

Net photosynthesis per unit leaf dry weight per hour was calculated after Williams and Kemp (1959) equations where

$$\text{Net Photosynthesis} = \frac{\Delta \text{CO}_2 \text{at } 14.01 \text{ g CO}_2/\text{mole} \times \frac{273^\circ\text{K}}{273^\circ\text{K} + T}}{1.8 \text{ g} \times \frac{1 \text{ liter}}{10^3 \text{ m}^3 \text{ liter}} \times \frac{60 \text{ min}}{\text{hr}} \times \frac{1 \text{ liter}}{10^3 \text{ m}^3 \text{ liter}} \times \frac{10^6 \text{ mg}}{\text{g}}}$$

( $\mu\text{g CO}_2 \text{ g}^{-1} \text{ hr}^{-1}$ )

where

$\Delta \text{CO}_2$  = Change in  $\text{CO}_2$  concentration ( $\mu\text{l/l}$ ) going through the cuvette.

$T$  = Flow rate of air through the cuvette (l/min).

1.8 = Dry leaf weight in grams.

Four to five plantlets and the same number of seedlings from each parent were randomly selected sampled at Harvests 2, 3, 4, and 5. Whole plant net photosynthesis measurement was taken for plants in Harvests 2 and 3 by enclosing the entire shoot system inside the cuvette, which isolated the root system. However, for Harvests 4 and 5 the plants were too big and only the top part of the shoot (with approximately 8-12 leaves) was enclosed inside the cuvette.

#### Chlorophyll Analysis

Chlorophyll was measured on the same plants which were sampled for photosynthesis. One-half of the foliage (along the entire length of the shoot which were measured for photosynthesis) of each plant for Harvest 2, 3, 4, and 5 was used for chlorophyll determination, and the other half for fresh and dry weight determination. Chlorophyll determination was based on that of Arnon (1949) on the absorption of light by aqueous acetone (80%) extracts of chlorophyll. Leaves were ground in liquid nitrogen and only 0.10 -0.15 g were added to five ml of freshly prepared 80% acetone. The suspension was then mixed and centrifuged at 4000 rpm for 15 minutes. One ml of the supernatant was then diluted to 5 ml with acetone (80%). Absorbance for chlorophyll A & B at 663 and 645 was measured by spectrophotometer. GCA (DuPont Instrument model E Q-700-

20) was used for Harvest 2 and 3, while Spectronic 20 by Milton Roy Company was used for Harvest 4 and 5. Chlorophyll content was calculated after Arnon ( 1948):

$$\text{Chlorophyll a (mg/g)} = (12.7\text{ODm}_{660} - 2.2\text{ODm}_{640}) / D \times \text{LF}$$

$$\text{Chlorophyll b (mg/g)} = (22.9\text{ODm}_{660} - 4.4\text{ODm}_{640}) / D \times \text{LF}$$

$$\text{Total chlorophyll (mg/g)} = (22.9\text{ODm}_{660} + 8.8\text{ODm}_{640}) / D \times \text{LF}$$

where

D = dilution factor ( 20 in this case),

LF = leaf fresh weight in grams.

#### Total Nitrogen Percent (%)

Total nitrogen percent was determined for stem, roots, and leaves of the same plants sampled for photosynthesis for Harvests 2,3,4, and 5. For Harvest 1 all the plants sampled for biomass data were sampled for total nitrogen percent. However because seedlings were very small, 5-6 seedlings from each family were combined together to form one sample unit. Plant tissues were digested with sulfuric acid and a catalyst (sulphur powder- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , $\text{HCl}$ ) for three hours at 170°C. They were then brought to volume with saline solution. Total nitrogen content was then determined as a colorimetric assay method in which an emerald-green color is formed by the reaction of ammonia, sodium salicylate, sodium nitroprusside, and sodium hypochlorite in a buffered, alkaline medium at a pH of 11.8 - 12.0. The ammonia-salicylate complex was read at 410 nm.

### Dry Matter Accumulation and Distribution

One plant per block was selected randomly (total of four) and destructively sampled for biomass parameters on harvest 1 through harvest 5. Plants were washed carefully and separated into stem, leaves, and roots and oven dry weight were taken. Ratios of oven dry weight of stem:root, leaf:stem:root, and shoot:root were then calculated. Root accretion was (per leaf dry weight) approximated after Weiss and Cassman (1978). Relative stem, leaves, shoot, root, and whole plant growth rates between harvests were also calculated after Weiss and Cassman (1978) as follows:

$$R = (\text{Average } \log_e W_2 - \text{Average } \log_e W_1) \div 1/(t_2 - t_1).$$

$$\text{Variance} = (s^2 \log_e W_2 + s^2 \log_e W_1) \div 1/(t_2 - t_1)^2$$

where

R = Relative growth rate per day.

$W_2 - W_1$  = Oven dry weight between each two harvests.

$t_2 - t_1$  = Number of days between each two harvests.

### Statistical Analysis

The statistical system SAS (1985) was used for all data analyses except the survival data. First plantlets and seedlings were analyzed separately at each harvest using GLM procedures (1985) for the analysis of variance for every variable (Y) with the following model:

$$Y_{ij} = \mu + \text{Parent}_i + \text{Block}_j + (\text{Parent} \times \text{Block})_{ij} + \text{Error}.$$

Parents were considered fixed and blocks random. Blocks and block x Parent interaction were found not significant for all the variables and so block effect was excluded from subsequent analyses and will not be discussed.

Secondly, harvest effect was analyzed for plantlets and seedlings separately with SAS (1988) GLM procedure for every variable except dry weight and relative growth rate with the following model:

$$Y_{ijk} = \mu + \text{Parent}_i + \text{Harvest}_j + (\text{Parent} \times \text{Harvest})_{ij} + \text{Error}_{ijk}$$

With Parents as fixed and Harvests as random, the Duncan's multiple range available in the means option in the GLM procedure was used to test the means for parent and harvests. Third, all plantlets and seedlings were compared at each harvest using the TTEST procedure in SAS (1988) for every variable. Fourth, multiple (stepwise, forward, and backward) and individual regression analysis for photosynthesis with foliar nitrogen, type [plantlets or seedling], and chlorophyll a and b, and total was performed with the REG procedure in SAS (1988). In all of the analyses significance was estimated at the 5% level.

## Results

### Survival

Plantlets and seedlings had high survival and did not differ significantly. At Harvest 2, survival was not nor

plantlets and 88% for seedlings, across the three parents, and increased to 100% for the other harvests.

### Net Photosynthesis

Significant variation was observed between harvests for both plantlets and seedlings (table 3-1). Seedling CO<sub>2</sub> fixation rate was significantly higher after the first forty days in greenhouse, at Harvest 1, and dropped sharply at Harvest 2 and then increased insignificantly at Harvest 4 and 5 (Figure 3-1). A similar pattern was observed with plantlets but the increase at Harvest 4 was significant. The interaction between the three parents and harvests and the main effect of parents were not significant for seedlings or plantlets for net photosynthesis.

Seedlings had significantly higher net photosynthesis at Harvest 2 than plantlets (table 3-1). However, by Harvest 5 the difference diminished and plantlets attained a rate similar to seedlings.

### Chlorophyll Content

Similar to net photosynthesis, the interaction between the three parents and harvests and the parent main effect were not significant among plantlets or seedlings for chlorophyll a, b, total chlorophyll per unit leaf fresh weight, and ratio of chlorophyll a:b. However, significant changes were observed with harvests (table 3-2). Plantlet

Table 3-1. Plantlet and seedling average net photosynthesis ( $\mu\text{g CO}_2 \text{ g}^{-1}$  leaf dry weight  $\text{h}^{-1}$ ) at harvests 2, 3, 4, and 5.

| Harvest | net photosynthesis  |                    |
|---------|---------------------|--------------------|
|         | plantlets           | seedlings          |
| 2       | 45.58 <sup>ab</sup> | 78.18 <sup>a</sup> |
| 3       | 56.62 <sup>b</sup>  | 38.42 <sup>b</sup> |
| 4       | 38.17 <sup>ab</sup> | 32.78 <sup>b</sup> |
| 5       | 27.71 <sup>ab</sup> | 32.38 <sup>b</sup> |

<sup>a</sup> Means with the same letters in the same column are not significantly different at the 5% level with Duncan's multiple range test.

<sup>b</sup> Indicates significant difference between plantlets and seedlings at Harvest 5.

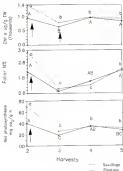


Figure 2-1. Plantlet and seedling chlorophyll a in  $\text{mg g}^{-1}$  from leaf weight (upper), total foliar nitrogen percent (middle) and net photosynthesis in  $\text{mg CO}_2 \text{g}^{-1} \text{h}^{-1}$  (lower) over harvests.

[Arrows indicate significant differences between plantlets and seedlings and means with same letters indicate no significantly different at 5% level]

Table 2-3. Plantlet and seedling mass chlorophyll *a*, *b*, and total (a+b) content (mg g<sup>-1</sup> leaf fresh weight) and chlorophyll *a:b* ratios at harvests 1, 2, 3, and 4.

|                              |  | Harvests             |                     |                     |                      |
|------------------------------|--|----------------------|---------------------|---------------------|----------------------|
|                              |  | 1                    | 2                   | 3                   | 4                    |
| Chlorophyll <i>a</i>         |  |                      |                     |                     |                      |
| Plantlets                    |  | 818.32 <sup>ab</sup> | 823.23 <sup>b</sup> | 848.79 <sup>c</sup> | 828.84 <sup>b</sup>  |
| Seedlings                    |  | 1382.8 <sup>a</sup>  | 781.1 <sup>b</sup>  | 941.4 <sup>b</sup>  | 889.8 <sup>b</sup>   |
| T test                       |  | *                    | *                   | -                   | -                    |
| Chlorophyll <i>b</i>         |  |                      |                     |                     |                      |
| Plantlets                    |  | 283.88 <sup>a</sup>  | 334.82 <sup>a</sup> | 393.88 <sup>a</sup> | 488.84 <sup>b</sup>  |
| Seedlings                    |  | 484.14 <sup>a</sup>  | 381.81 <sup>a</sup> | 543.13 <sup>a</sup> | 484.39 <sup>a</sup>  |
| T test                       |  | *                    | -                   | -                   | -                    |
| Chlorophyll <i>a:b</i>       |  |                      |                     |                     |                      |
| Plantlets                    |  | 2839.8 <sup>a</sup>  | 2448.9 <sup>a</sup> | 2144.5 <sup>a</sup> | 1678.4 <sup>b</sup>  |
| Seedlings                    |  | 2876.8 <sup>a</sup>  | 2023.7 <sup>a</sup> | 1682.2 <sup>a</sup> | 1803.7 <sup>ab</sup> |
| T test                       |  | *                    | *                   | -                   | -                    |
| Chlorophyll <i>a:b</i> ratio |  |                      |                     |                     |                      |
| Plantlets                    |  | 3.84 <sup>a</sup>    | 3.84 <sup>a</sup>   | 3.71 <sup>a</sup>   | 3.46 <sup>b</sup>    |
| Seedlings                    |  | 3.85 <sup>a</sup>    | 3.85 <sup>a</sup>   | 3.78 <sup>a</sup>   | 3.84 <sup>a</sup>    |
| T test                       |  | -                    | -                   | -                   | -                    |

\* Indicates significant difference between plantlets and seedlings for the indicated harvest at 5% level.

a/ Means with the same letters in the same row are not significantly different according to Duncan's multiple range test.

chlorophyll a content dropped significantly at Harvest 3 and increased significantly at Harvest 4 and 5 (Table 3-2). Seedlings showed a similar but insignificant pattern. Total chlorophyll showed similar pattern with significantly lower content at harvest 3 for both plantlets and seedlings (Table 3-2). Chlorophyll b, on the other hand, tended to increase with harvest except at Harvest 3 (Table 3-2, Figure 3-3). Plantlet chlorophyll b content increased significantly at Harvest 4 and 5. The decrease in chlorophyll a and increase in chlorophyll b was reflected in a significant drop of chlorophyll a/b ratio with harvest for plantlets and seedlings alike (Table 3-2, Figure 3-3).

Similar to net photosynthesis, seedlings had significantly higher chlorophyll a, b, and total chlorophyll at early harvests than plantlets, and the difference decreased with time. However, chlorophyll a/b ratio of plantlets and seedlings was not significantly different when compared at each harvest (Table 3.2).

#### Total Nitrogen Percent (Nt)

Similar to net photosynthesis and chlorophyll contents, the interaction between the three parents and harvests and the parent main effect were not significant for seedlings or plantlets for leaf, stem, and root total Nt. However, Nt changed significantly with harvests (Table 3-3). Leaf Nt dropped significantly at Harvest 3 and then

Table 2-3. Plantlet and seedling mean leaf, stem, and root total nitrogen percent (Nt) for harvests 1, 2, 3, 4, and 5.

|                              | Harvests          |                   |                    |                   |                   |
|------------------------------|-------------------|-------------------|--------------------|-------------------|-------------------|
|                              | 1                 | 2                 | 3                  | 4                 | 5                 |
| <b>Leaf Nt</b>               |                   |                   |                    |                   |                   |
| Plantlets 2.38 <sup>ab</sup> | 2.13 <sup>a</sup> | 2.82 <sup>b</sup> | 1.78 <sup>ab</sup> | 2.33 <sup>a</sup> | 2.33 <sup>a</sup> |
| Seedlings 2.88 <sup>a</sup>  | 2.47 <sup>a</sup> | 3.38 <sup>a</sup> | 1.83 <sup>a</sup>  | 2.37 <sup>a</sup> | 2.37 <sup>a</sup> |
| T test                       | *                 | *                 | -                  | -                 | -                 |
| <b>Stem Nt</b>               |                   |                   |                    |                   |                   |
| Plantlets 1.38 <sup>a</sup>  | 1.20 <sup>a</sup> | 0.42 <sup>b</sup> | 0.38 <sup>b</sup>  | 0.38 <sup>b</sup> | 0.38 <sup>b</sup> |
| Seedlings 1.42 <sup>a</sup>  | 1.08 <sup>a</sup> | 0.42 <sup>b</sup> | 0.42 <sup>b</sup>  | 0.38 <sup>b</sup> | 0.38 <sup>b</sup> |
| T test                       | *                 | *                 | -                  | -                 | -                 |
| <b>Root Nt</b>               |                   |                   |                    |                   |                   |
| Plantlets 1.38 <sup>a</sup>  | 1.08 <sup>a</sup> | 0.42 <sup>b</sup> | 0.38 <sup>b</sup>  | 0.38 <sup>b</sup> | 0.38 <sup>b</sup> |
| Seedlings 1.42 <sup>a</sup>  | 1.27 <sup>a</sup> | 0.42 <sup>b</sup> | 0.42 <sup>b</sup>  | 0.42 <sup>b</sup> | 0.42 <sup>b</sup> |
| T test                       | *                 | *                 | -                  | -                 | -                 |

\* Indicates significant difference between plantlets and seedlings at the 5% level.

† Means with the same letter on the same row are not different according to Duncan's multiple range test at 5% level.

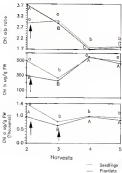


Figure 3-2. Plantlet and seedling chlorophyll *a* (top), chlorophyll *b* (middle) and *a* (lower) in mg/g leaf fresh weight over harvests.

(Arrows indicate significant differences between plantlets and seedlings and means with same letters are not significantly different at 0.1 level)

increased significantly by Harvest 3 for plantlets and seedlings. Root and stem N continued to drop significantly with time for both seedlings and plantlets.

Also similar to net photosynthesis and chlorophyll content, seedlings had significantly higher total N in their leaves, stem, and roots at early harvests than plantlets. By Harvest 3 plantlets had similar leaf, root, and stem total nitrogen percent as seedlings (Table 3-3).

#### Net Photosynthesis, Chlorophyll and Foliar N

Net photosynthesis, chlorophyll, and foliar total N dropped sharply at Harvest 3 (Tables 3-4 to 3-6, Figures 3-1). That was preceded by high relative growth rates between Harvest 1-2 and 2-3 (Table 3-13, Figure 3-13). Multiple and individual regression analysis showed that net photosynthesis is more correlated with foliar N and chlorophyll a.

Stepwise, forward, and backward regression of net photosynthesis with chlorophyll a, b and a+b, foliar nitrogen, and origin (seedling or plantlet) showed only foliar N and chlorophyll a were significant in the model ( $p < .05$  level). Chlorophyll b and total chlorophyll, and origin were not significant even at the the 0.1% level. origin (seedling or seedling) had the least effect in the model. N was highly correlated with net photosynthesis and was entered first in the stepwise regression yielding the

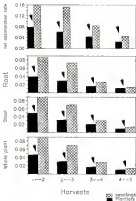


Figure 3-3. Plantlet and seedling self-assimilation rate (Upper) and relative growth rates per day of root, shoot and whole plant (lower) between harvests.

(Arrows indicate significant difference between plantlets and seedlings the 5% level)

following relationships:

$$\text{Net photosynthesis} = 10.82 + 12.766 \text{ chl } a \quad (R^2 = 0.4781),$$

Chlorophyll *a* (chl *a*) was entered second in the model with

$$\text{Net photosynthesis} = 1.82 + 0.015 \text{ chl } a + 10.82 \text{ R } 2$$

$$(R^2 = 0.517 \text{ and partial } R^2 \text{ of } 0.04 \text{ for chl } a).$$

Individual regression of net photosynthesis on total foliar nitrogen percent (NF), chlorophyll (chl) *a*, *b*, and total separately yielded significant (at 0.05 level) models (Table 3-4). Total foliar nitrogen showed higher correlation with  $R^2 = 0.11$ , while chlorophyll *a*, *b* and total had  $R^2$  of 0.03, 0.076, and 0.104, respectively.

Regression of net photosynthesis on log chlorophyll *a*, *b*, and total did not improve  $R^2$  however excluding higher net photosynthesis values (more than 28 mg CO<sub>2</sub> per g leaf dry weight per hour) improved  $R^2$  considerably (Table 3-4).

## DRY-WEIGHT ACCUMULATION AND DISTRIBUTION

### Plantlets versus seedlings

Plantlets started with significantly higher stem, leaf, shoot, root, and total plant weights compared to seedlings (Table 3-5). These differences decreased with harvest, but continued to be significant through harvest 5, except for stem weight which was not significantly different at Harvest 4 and 5. The largest initial difference was observed with root weight. Plantlet:seedling

Table 3-4. Individual regression models for net photosynthesis ( $\text{mg CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ ) with foliar N, and Chlorophyll (chl) a, b, and total.

| Dependent Variable       | Independent Variable | $b_0$ | $b_1$ | $R^2$ |
|--------------------------|----------------------|-------|-------|-------|
| Net photo.1 <sup>1</sup> | Foliar N             | 4.84  | 15.67 | 0.59  |
| Net photo.1              | chl a                | 4.9   | 0.029 | 0.25  |
| Net photo.1              | chl b                | 12.48 | 0.03  | 0.84  |
| Net photo.1              | chl total            | 12.88 | 0.008 | 0.29  |
| Net photo.2              | chl a                | 8.43  | 0.003 | 0.60  |
| Net photo.2              | chl b                | 14.72 | 0.003 | 0.43  |
| net photo.2              | chl total            | 8.69  | 0.004 | 0.53  |

<sup>1</sup> Net photo.1 indicates all net photosynthesis values were used in developing the regression model while net photo.2 indicates that values higher than 80  $\text{mg CO}_2 \text{ g}^{-1} \text{ h}^{-1}$  were excluded.

Table 3-6. Plantlet and seedling mean stem, leaf, shoot, root, and total dry weight in grams and plantlet:seedling ratio at each harvest.

| Parameter    | Harvest |        |        |       |       |
|--------------|---------|--------|--------|-------|-------|
|              | 1       | 2      | 3      | 4     | 5     |
| <b>Stem</b>  |         |        |        |       |       |
| Plantlets    | 0.002   | 0.003  | 0.408  | 1.198 | 0.394 |
| Seedlings    | 0.0003  | 0.018  | 0.308  | 1.003 | 1.030 |
| T test       | *       | *      | *      | *     | *     |
| Ratio        | 40      | 0.3    | 1.40   | 1.20  | 1.21  |
| <b>Leaf</b>  |         |        |        |       |       |
| Plantlets    | 0.0373  | 0.243  | 0.683  | 1.333 | 1.038 |
| Seedlings    | 0.0005  | 0.039  | 0.332  | 0.645 | 1.033 |
| T test       | *       | *      | *      | *     | *     |
| Ratio        | 76      | 6      | 2.06   | 1.018 | 1.68  |
| <b>Shoot</b> |         |        |        |       |       |
| Plantlets    | 0.0493  | 0.2843 | 0.3094 | 2.433 | 3.49  |
| Seedlings    | 0.0008  | 0.0383 | 0.88   | 1.648 | 3.67  |
| T test       | *       | *      | *      | *     | *     |
| Ratio        | 61.8    | 0.66   | 2.60   | 1.48  | 1.24  |
| <b>Roots</b> |         |        |        |       |       |
| Plantlets    | 0.0201  | 0.100  | 0.327  | 0.450 | 1.093 |
| Seedlings    | 0.0003  | 0.008  | 0.344  | 0.378 | 0.694 |
| T test       | *       | *      | *      | *     | *     |
| Ratio        | 58-5    | 12.88  | 0.37   | 1.74  | 1.60  |
| <b>Total</b> |         |        |        |       |       |
| Plantlets    | 0.076   | 0.437  | 1.430  | 1.084 | 4.783 |
| Seedlings    | 0.001   | 0.047  | 0.684  | 0.643 | 3.453 |
| T test       | *       | *      | *      | *     | *     |
| Ratio        | 78      | 9.40   | 2.13   | 1.68  | 1.51  |

\* indicates significant difference at the 5% level between plantlets and seedlings at the same harvest.

root weight ratio at Harvest 1 was higher than that of stem, leaves, shoot, and total, but it dropped sharply from 110 at Harvest 1 to 12.88 by Harvest 2 and continued dropping to 1.8 at Harvest 3 (Table 3-3). Plantlet/seedling stem weight ratio, on the other hand, dropped from 48 at Harvest 1 to 1.11 at Harvest 3. A similar pattern was also observed with leaf weight where plantlet/seedling ratio was 74 at Harvest 1 and 1.48 at Harvest 3.

Ratio of plantlet above ground to below ground components showed more accumulation in the below ground as compared to seedlings. Plantlets had significantly lower stem:root, shoot:root, and leaves:root ratios than seedlings (Table 3-4, Figure 3-4), with the largest difference at Harvest 1.

Average relative growth rates (Table 3-13, Figure 3-5) showed that plantlets had lower rates than seedlings. Relative growth rate for stem, leaves, shoots, and whole plant decreased with time for both plantlets and seedlings, with a higher drop in seedlings.

Net assimilation rates per leaf dry weight (Figure 3-5) showed similar response as the relative growth rates. Seedlings had significantly higher rates than plantlets through the five harvests and the differences decreased slightly with time.

### Parent effect

Parent effect analyzed separately for plantlets and seedlings at each harvest showed significant differences in most cases among seedlings and plantlets (Tables 3-7 to 3-11).

At Harvest 1, no significant differences in total biomass were detected among the seedlings. However considerable differences were observed in the pattern of growth (Tables 3-7). Parent 2417 seedlings had significantly higher stem biomass, while parent 2798 seedlings had significantly higher root biomass. This also resulted in significantly higher stem:root and shoot:root ratios for parent 2417. Plantlets, on the other hand, showed no significant variation was observed between plantlets after the hardening process, neither in the amount nor in the pattern of biomass accumulation between the three parents.

Unlike Harvest 1, Harvest 2 showed significant variation between plantlets for the three parents while no differences were observed for the seedlings in the pattern of biomass accumulation (Table 3-8). Similar to Harvest 1, no significant variation was observed for total biomass between plantlets; however, allocation of dry matter was different. Plantlets showed similar allocation patterns as seen with seedlings in Harvest 1 as parent 2417 had significantly higher stem biomass and parent 2798 had higher root biomass. Although no significant variation was

Table 3-4. Plantlet and seedling mass abovebelow ground dry weight ratios (stem:root, leaf:root, and shoot:root) at each harvest.

| Ratio                   | Harvest |       |       |       |       |
|-------------------------|---------|-------|-------|-------|-------|
|                         | 1       | 2     | 3     | 4     | 5     |
| <b>Stem:root</b>        |         |       |       |       |       |
| Plantlets               | 0.587   | 0.669 | 0.594 | 1.009 | 0.883 |
| Seedlings               | 1.018   | 1.454 | 0.869 | 0.947 | 0.845 |
| T test                  | *       | *     | *     | *     | *     |
| Ratio                   | 0.71    | 0.47  | 0.88  | 0.94  | 0.98  |
| <b>Leaf:root ratio</b>  |         |       |       |       |       |
| Plantlets               | 0.883   | 0.876 | 0.118 | 1.044 | 1.418 |
| Seedlings               | 0.644   | 0.877 | 0.402 | 0.218 | 1.892 |
| T test                  | *       | *     | *     | *     | *     |
| Ratio                   | 0.43    | 0.67  | 0.88  | 0.88  | 0.74  |
| <b>Shoot:root ratio</b> |         |       |       |       |       |
| Plantlets               | 0.181   | 0.648 | 0.821 | 1.013 | 0.868 |
| Seedlings               | 0.691   | 0.712 | 0.818 | 0.948 | 0.872 |
| T test                  | *       | *     | *     | *     | *     |
| Ratio                   | 0.43    | 0.61  | 0.67  | 0.49  | 0.84  |

\* indicates significant difference between plantlets and seedlings at the 5% level.

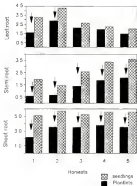


Figure 2-4. Plantlet and seedling leaf:root (upper), stem:root (middle) and shoot:root (lower) ratios over harvests.

(Arrows indicate significant difference between plantlets and seedlings at the 5% level)

Table 3-7. Flaxseed and seedling mean dry weights and their ratios for Harvest 1 biomass parameters of three parents.

| Variable   | Flaxseeds            |                     |                     | Seedlings           |                     |                     |
|------------|----------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|            | 2017                 | 2018                | 2019                | 2017                | 2018                | 2019                |
| Seed(g)    | 0.0077 <sup>ab</sup> | 0.0124 <sup>a</sup> | 0.0210 <sup>a</sup> | 0.0004 <sup>d</sup> | 0.0003 <sup>b</sup> | 0.0003 <sup>d</sup> |
| Leaf(g)    | 0.0020 <sup>b</sup>  | 0.0036 <sup>a</sup> | 0.0015 <sup>d</sup> | 0.0006 <sup>c</sup> | 0.0003 <sup>a</sup> | 0.0004 <sup>c</sup> |
| Shoot(g)   | 0.0097 <sup>a</sup>  | 0.0161 <sup>a</sup> | 0.0225 <sup>a</sup> | 0.0010 <sup>c</sup> | 0.0006 <sup>a</sup> | 0.0007 <sup>d</sup> |
| Root(g)    | 0.0018 <sup>d</sup>  | 0.0027 <sup>c</sup> | 0.0050 <sup>b</sup> | 0.0002 <sup>d</sup> | 0.0002 <sup>b</sup> | 0.0004 <sup>c</sup> |
| Total(g)   | 0.0115 <sup>a</sup>  | 0.0188 <sup>a</sup> | 0.0275 <sup>a</sup> | 0.0012 <sup>c</sup> | 0.0008 <sup>a</sup> | 0.0011 <sup>d</sup> |
| Seed:Root  | 0.38 <sup>a</sup>    | 0.56 <sup>a</sup>   | 0.38 <sup>a</sup>   | 0.51 <sup>a</sup>   | 1.01 <sup>b</sup>   | 1.20 <sup>b</sup>   |
| Shoot:Root | 5.45 <sup>a</sup>    | 5.94 <sup>a</sup>   | 4.51 <sup>a</sup>   | 5.01 <sup>a</sup>   | 7.30 <sup>b</sup>   | 3.00 <sup>a</sup>   |

^ Means with the same letter (for the same row and type (Flaxseeds or Seedlings)) are not significantly different at 5% level with Duncan's multiple range test.

Table 3-4. Flaxseed and seedling mean dry weights and their ratios for Harvest 3 biomass parameters of three parents.

| Variables  | Flaxseeds           |                     |                     | Seedlings          |                    |                    |
|------------|---------------------|---------------------|---------------------|--------------------|--------------------|--------------------|
|            | 2827                | 2814                | 2798                | 2817               | 2828               | 2798               |
| Stem (g)   | 0.0832 <sup>a</sup> | 0.0826 <sup>a</sup> | 0.0881 <sup>a</sup> | 0.012 <sup>a</sup> | 0.008 <sup>a</sup> | 0.008 <sup>a</sup> |
| Leaf (g)   | 0.2703 <sup>a</sup> | 0.2821 <sup>a</sup> | 0.2347 <sup>a</sup> | 0.034 <sup>a</sup> | 0.030 <sup>a</sup> | 0.033 <sup>a</sup> |
| Shoot (g)  | 0.3535 <sup>a</sup> | 0.3647 <sup>a</sup> | 0.3228 <sup>a</sup> | 0.046 <sup>a</sup> | 0.038 <sup>a</sup> | 0.041 <sup>a</sup> |
| Root (g)   | 0.3038 <sup>a</sup> | 0.1188 <sup>a</sup> | 0.0882 <sup>a</sup> | 0.009 <sup>a</sup> | 0.008 <sup>a</sup> | 0.009 <sup>a</sup> |
| Total (g)  | 0.6573 <sup>a</sup> | 0.4835 <sup>a</sup> | 0.4110 <sup>a</sup> | 0.055 <sup>a</sup> | 0.046 <sup>a</sup> | 0.050 <sup>a</sup> |
| Stem:Root  | 0.849 <sup>a</sup>  | 0.581 <sup>a</sup>  | 0.443 <sup>a</sup>  | 1.555 <sup>a</sup> | 1.393 <sup>a</sup> | 0.457 <sup>a</sup> |
| Shoot:Root | 4.127 <sup>a</sup>  | 4.079 <sup>a</sup>  | 3.734 <sup>a</sup>  | 6.159 <sup>a</sup> | 5.640 <sup>a</sup> | 5.115 <sup>a</sup> |

\. Means with the same letter for the same row and type (Flaxseed or Seedlings) are not significantly different at 5% level with Duncan's multiple range test.

Table 3-6. Plantlet and seedling mean dry weights and their ratios for March 3 biomass parameters of three parents.

| Parameter  | Plantlets          |                    |                    | Seedlings          |                     |                    |
|------------|--------------------|--------------------|--------------------|--------------------|---------------------|--------------------|
|            | 2013               | 2014               | 2016               | 2013               | 2014                | 2016               |
| Stem (g)   | 0.312 <sup>a</sup> | 0.391 <sup>a</sup> | 0.279 <sup>a</sup> | 0.323 <sup>a</sup> | 0.243 <sup>a</sup>  | 0.327 <sup>a</sup> |
| Leaf (g)   | 0.399 <sup>a</sup> | 0.434 <sup>a</sup> | 0.488 <sup>a</sup> | 0.333 <sup>a</sup> | 0.243 <sup>a</sup>  | 0.343 <sup>a</sup> |
| Shoot (g)  | 1.045 <sup>a</sup> | 0.789 <sup>a</sup> | 0.741 <sup>a</sup> | 0.666 <sup>a</sup> | 0.514 <sup>a</sup>  | 0.629 <sup>a</sup> |
| Root (g)   | 0.342 <sup>a</sup> | 0.331 <sup>a</sup> | 0.372 <sup>a</sup> | 0.149 <sup>a</sup> | 0.138 <sup>a</sup>  | 0.158 <sup>a</sup> |
| Total (g)  | 1.438 <sup>a</sup> | 0.995 <sup>a</sup> | 1.034 <sup>a</sup> | 0.834 <sup>a</sup> | 0.644 <sup>a</sup>  | 0.793 <sup>a</sup> |
| Stem:Root  | 1.932 <sup>a</sup> | 2.334 <sup>a</sup> | 0.741 <sup>a</sup> | 2.133 <sup>a</sup> | 3.638 <sup>ab</sup> | 1.718 <sup>a</sup> |
| Shoot:Root | 3.023 <sup>a</sup> | 2.431 <sup>a</sup> | 1.931 <sup>a</sup> | 4.443 <sup>a</sup> | 3.653 <sup>ab</sup> | 3.733 <sup>a</sup> |

<sup>a</sup> Means with the same letter (for the same year and type (Plantlets or Seedlings)) are not significantly different at 5% level with Duncan's multiple range test.

Table 3-18. Flaxseed and seedling mean dry weights and ratios (air) for Harvest 4 biomass parameters of three parents.

| Parameter  | Flaxseeds           |                    |                     | Seedlings          |                     |                    |
|------------|---------------------|--------------------|---------------------|--------------------|---------------------|--------------------|
|            | 2017                | 2018               | 2019                | 2017               | 2018                | 2019               |
| Seed (g)   | 1.770 <sup>ab</sup> | 1.697 <sup>b</sup> | 0.860 <sup>b</sup>  | 2.117 <sup>a</sup> | 0.999 <sup>c</sup>  | 0.931 <sup>c</sup> |
| Leaf (g)   | 1.466 <sup>a</sup>  | 1.505 <sup>b</sup> | 1.182 <sup>ab</sup> | 0.878 <sup>a</sup> | 0.838 <sup>a</sup>  | 0.882 <sup>a</sup> |
| Shoot (g)  | 3.136 <sup>a</sup>  | 3.202 <sup>a</sup> | 2.042 <sup>b</sup>  | 2.991 <sup>a</sup> | 1.837 <sup>a</sup>  | 1.813 <sup>a</sup> |
| Root (g)   | 0.761 <sup>b</sup>  | 0.879 <sup>a</sup> | 0.603 <sup>c</sup>  | 0.314 <sup>b</sup> | 0.134 <sup>b</sup>  | 0.460 <sup>a</sup> |
| Total (g)  | 3.897 <sup>a</sup>  | 4.073 <sup>a</sup> | 2.645 <sup>b</sup>  | 3.305 <sup>a</sup> | 1.971 <sup>a</sup>  | 2.273 <sup>a</sup> |
| Shoot:Root | 2.644 <sup>a</sup>  | 3.676 <sup>b</sup> | 3.402 <sup>c</sup>  | 9.518 <sup>a</sup> | 13.819 <sup>a</sup> | 3.939 <sup>b</sup> |
| Shoot:Seed | 1.785 <sup>a</sup>  | 1.935 <sup>a</sup> | 2.357 <sup>a</sup>  | 0.713 <sup>a</sup> | 0.199 <sup>a</sup>  | 1.031 <sup>a</sup> |

^ Means with the same letter for the same row and type (Flaxseeds or Seedlings) are not significantly different at 5% level with Duncan's multiple range test.

Table 3-11. Plantlet and seedling mean dry weights and ratios their for Harvest 3 biomass parameters of three parents.

| Parameter  | Plantlets          |                    |                    | Seedlings          |                    |                    |
|------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
|            | 2027               | 2028               | 2708               | 2027               | 2028               | 2708               |
| Stem (g)   | 1.808 <sup>a</sup> | 1.897 <sup>a</sup> | 1.719 <sup>a</sup> | 2.188 <sup>a</sup> | 1.974 <sup>a</sup> | 2.858 <sup>a</sup> |
| Leaf (g)   | 1.893 <sup>a</sup> | 2.907 <sup>a</sup> | 1.374 <sup>a</sup> | 1.897 <sup>a</sup> | 1.853 <sup>a</sup> | 2.949 <sup>a</sup> |
| Shoot (g)  | 3.701 <sup>a</sup> | 4.805 <sup>a</sup> | 3.093 <sup>a</sup> | 4.085 <sup>a</sup> | 3.828 <sup>a</sup> | 5.807 <sup>a</sup> |
| Root (g)   | 1.248 <sup>a</sup> | 0.737 <sup>a</sup> | 1.208 <sup>a</sup> | 0.508 <sup>a</sup> | 0.739 <sup>a</sup> | 0.733 <sup>a</sup> |
| Total (g)  | 4.949 <sup>a</sup> | 5.542 <sup>a</sup> | 4.301 <sup>a</sup> | 4.593 <sup>a</sup> | 4.568 <sup>a</sup> | 6.540 <sup>a</sup> |
| Stem/Root  | 1.449 <sup>a</sup> | 6.524 <sup>a</sup> | 2.515 <sup>a</sup> | 8.238 <sup>a</sup> | 5.198 <sup>a</sup> | 8.059 <sup>a</sup> |
| Shoot/Root | 4.007 <sup>a</sup> | 6.534 <sup>a</sup> | 2.238 <sup>a</sup> | 8.060 <sup>a</sup> | 6.299 <sup>a</sup> | 9.233 <sup>a</sup> |

^ Means with the same letter for the same row and type (Plantlets or Seedlings) are not significantly different at 5% level with Duncan's multiple range test.

Table 3-13. Plantlet and seedling relative stem, leaf, shoot, root, and whole plant growth rate per day between treatments.

| Parameter                               | Treatment   |             |             |             |
|-----------------------------------------|-------------|-------------|-------------|-------------|
|                                         | 1-2         | 2-3         | 3-4         | 4-5         |
| <b>Stem Relative Growth Rate</b>        |             |             |             |             |
| Plantlets                               | 0.042 (8.7) | 0.048 (8.4) | 0.024 (8.8) | 0.018 (7.5) |
| Seedlings                               | 0.077 (7.8) | 0.087 (18)  | 0.020 (8.4) | 0.014 (7.3) |
| T test                                  | *           | *           | *           | *           |
| Ratio                                   | 0.54        | 0.54        | 0.48        | 0.92        |
| <b>Leaf Relative Growth Rate</b>        |             |             |             |             |
| Plantlets                               | 0.002 (7)   | 0.009 (8)   | 0.018 (8)   | 0.0088 (8)  |
| Seedlings                               | 0.008 (8)   | 0.000 (18)  | 0.004 (8)   | 0.018 (7)   |
| T test                                  | *           | *           | *           | *           |
| Ratio                                   | 0.82        | 0.42        | 0.41        | 0.58        |
| <b>Shoot Relative Growth Rate</b>       |             |             |             |             |
| Plantlets                               | 0.445 (8.5) | 0.532 (34)  | 0.333 (34)  | 0.038 (8)   |
| Seedlings                               | 0.882 (8)   | 0.878 (38)  | 0.816 (8)   | 0.404 (7)   |
| T test                                  | *           | *           | *           | *           |
| Ratio                                   | 0.54        | 0.60        | 0.78        | 0.73        |
| <b>Root Relative Growth Rate</b>        |             |             |             |             |
| Plantlets                               | 0.000 (7)   | 0.038 (8)   | 0.017 (8)   | 0.038 (8)   |
| Seedlings                               | 0.088 (18)  | 0.072 (38)  | 0.024 (18)  | 0.038 (18)  |
| T test                                  | *           | *           | *           | *           |
| Ratio                                   | 0.46        | 0.48        | 0.44        | 0.48        |
| <b>Whole Plant Relative Growth Rate</b> |             |             |             |             |
| Plantlets                               | 0.847 (7)   | 0.934 (31)  | 0.938 (8)   | 0.821 (8)   |
| Seedlings                               | 0.890 (8)   | 0.878 (38)  | 0.938 (8)   | 0.854 (8)   |
| T test                                  | *           | *           | *           | *           |
| Ratio                                   | 0.98        | 0.44        | 0.43        | 0.76        |

\* indicates significant difference between plantlets and seedlings at the 95 level.

% Number in parenthesis indicates variance at 1000.

observed between seedlings, parents showed the same pattern displayed in Harvest 1. Plantlets were significantly different for their stem, root weight, and stem:root ratio. Parent 2817 had significantly higher stem weight and stem:root ratio, while parent 2798 had higher root weight and lower stem:root ratio. These patterns are similar pattern to seedlings within Harvest 1.

By Harvest 1, seedlings showed significant differences in their pattern of biomass distribution to shoot and roots (Table 3-5). While no significant differences were seen between total biomass of the three parents, parent 2817 showed higher above ground biomass while parent 2798 showed higher under ground biomass. Stem:root ratio was significantly higher for parent 2817. Plantlets, on the other hand, showed significant variation for stem, root, and total weight, and stem:root, and shoot:root ratios. Parent 2817 had higher above ground biomass compared to parents 2814 and 2798.

Similar results were observed with Harvests 4 and 5 (Table 3-14, 3-15), with pronounced differences between plantlets of the three parents. Parent 2817 had higher stem biomass, and consequently higher stem:root biomass followed by parent 2814 and then parent 2798. Total root biomass was not significantly different. Although no significant differences were observed for seedlings, parent 2817 showed higher above ground biomass and higher stem:root ratios.

In summary the biomass data showed similar patterns between plantlets and seedlings of the same parent. Parent 1007 had high above ground:below ground ratio, whereas parent 1178 exhibited a low ratio and parent 1814 had an intermediate value.

### Discussion

One of the main problems of comparing plantlets with seedlings is differences in physiological and developmental age. This study used a functional approach similar to that of McDaniel and Allen (1984) where plantlets and seedlings were compared from the time they came out of mist-greenhouses and they were ready for normal greenhouse conditions. Results of this study about survival, dry matter accumulation, allocation to roots, shoots, leaves, photosynthetic rates and total nitrogen percent support some hypotheses.

#### Survival

Unlike the hypothesis, survival of plantlets was not significantly different from that of seedlings, where 100 and 100% survival was observed for plantlets and seedlings respectively, through Harvest 3 across the three parents. The high plantlet survival rate may indicate that the previous steps in culture, especially the elongation step discussed in the previous chapter, produced more adapted

plantlets better able to withstand normal greenhouse conditions. Survival of plantlets under normal conditions is one of the most limiting factors for any tissue culture based operation. Thus, the high survival displayed by plantlets from very early to their possible use for operational planting. High mortality of 10% was observed with labially pine plantlets (Lensch, 1974). However, labially pine plantlets were reported to have significantly less biomass and root:shoot ratio as compared to seedlings (Lensch 1974, McDonald and Allen, 1984). The opposite was observed in this study with *P. grandis* plantlets and seedlings. These two factors, especially root:shoot ratio, may have contributed to the high survival of *P. grandis* plantlets as compared to those of labially pine. Another factor is the high net photosynthesis observed with plantlets, even at early stages of development (Harvest 3) (Table 3-1).

#### Net Photosynthesis, Chlorophyll and Total N

In agreement with the hypotheses, plantlet net photosynthesis, chlorophyll content and total nitrogen percent were significantly different from seedlings at early harvests. By Harvest 4 no significant differences were observed between them (Tables 3-1 to 3-3). The results also showed that seedling age and foliar nitrogen content were very important factors in determining net

photosynthesis efficiency per dry leaf weight. In addition, seedlings and plantlets showed the similar trend over harvests, with net photosynthesis values close to those reported for other young *Passiflora* seedlings.

One of the major concerns about tissue-culture-derived plantlets is their photosynthetic capabilities. It was reported that culture condition reduce autotrophic metabolism in vitro isolated cell culture (Bassmann, 1983) and plantlets under culture conditions (Gennelly and Vidaver 1984) or post culture (Brook and Aston, 1988; Gennelly and Vidaver, 1984). Seedlings had significantly higher net photosynthesis than plantlets only at Harvest 1. The difference at Harvest 1 seems to be related to seedling age rather than effect of the culture condition on plantlets.

Considering only plantlets, if there is any carry over effect from culture environment, it should show more at early harvests. However, net photosynthesis at Harvest 1 was either higher or equal to that of other harvests (Table 1-1, Figure 2-3). That would indicate that either culture condition had no carry-over effect on post-culture net photosynthesis or it lasted only a short time and that was missed, because plantlets were sampled 45 days after the hardening step. Brook and Milnes (1988) reported increased net photosynthesis for strawberry plantlets 14 days after transplanting. Due to the direction of new leaves, net

affected by culture conditions, and loss of leaves produced under in vitro conditions. That may be true in this study, although, no apparent loss of leaves was observed in the first 40 days, a considerable number of new leaves were produced within those 40 days. In any case, the practical significance of the high photosynthetic rate, even at early stages (Harvest 1), may have contributed to the high survival rate observed with plantlets. Net photosynthetic values observed with plantlets at any harvest in this study were higher or equal to that reported by Mooney et al. (1978) for seven *Eucalyptus* species seedlings.

On the other hand, exceptionally high net photosynthesis for seedlings at Harvest 2 can be linked to plant age. Lorton and Gordon (1965) reported higher photosynthesis efficiency for supplementary and first leaves of young seedlings. Similarly Mooney et al. (1978) observed that younger *Eucalyptus* seedlings had photosynthesis rates that were generally at least twice that of older nursery seedlings. Likewise, in this study 8-week-old seedlings (Harvest 2) had 1.8 times more net photosynthesis per unit dry weight than 22-week-old seedlings (Harvest 3) and almost twice that for 37- and 43-week-old seedlings (Harvests 4 and 5).

Another important result is the that plantlets were parallel to seedlings in net photosynthesis, chlorophyll and total foliar nitrogen over time (Figure 3-4). This may

reflect that there was no change in the plantlet basic physiological response, in term of these variation, to changing conditions around plantlets and seedlings.

Net photosynthesis was affected mainly by foliar nitrogen and chlorophyll *a*. origin (seedling or plantlet) was highly insignificant in the multiple regression model. Net photosynthesis was highly correlated ( $r = 0.7$ ) with foliar nitrogen percent of both plantlets and seedlings, similar to other species like *Rhus mixta* (Jeong and Whitt, 1981), *Eucalyptus* (Mooney et al., 1988), and peach (Jeong, 1981). The higher correlation with nitrogen rather than chlorophyll, which is necessary for capturing radiant energy in photosynthesis, is because nitrogen is included in ribulose biphosphate carboxylase enzymes, which makes up a substantial fraction of the total leaf proteins (Mooney and Gulmon, 1978). Also nitrogen can be viewed as an expensive limiting element, so it is logical that it has a positive high correlation with net photosynthesis. The high seedling foliar nitrogen percent of Harvest 3 as compared to Harvest 1, 4, and 5 (Table 2-3) may explain the high seedling net photosynthesis at Harvest 3.

Chlorophyll content, on the other hand, was not as highly correlated with net photosynthesis, except when higher values of net photosynthesis were excluded (Table 2-4). Similar results were mentioned by Binder and Book (1984) with other species. They explained that since

chlorophyll is usually considered to be a non-limiting factor unless plants are under nutrient stress, which may result in low photosynthetic rate. This may explain the higher correlation obtained when higher net photosynthetic values were excluded from the regression analysis.

### Dry Matter Accumulation and Relative Distribution

#### Plantlets versus seedlings

stem, leaf, shoot (stem plus leaves), root and whole plant dry weight differed significantly between plantlets and seedlings, as hypothesized. Plantlets maintained significantly higher weight through Harvest 1 (Table 3-5). However, these differences dropped sharply with subsequent harvests (Table 3-5). The main reason is the higher relative growth rate observed with seedlings (Table 3-12). The largest difference, root dry weight at Harvest 1 where plantlets weighed 130 times more than seedlings, may indicate that at the hardening step plantlets accumulated more dry matter in their root system as a stress response (Clemitt, 1988). These results were in contrast to loblolly pine plantlets (Leach, 1978; McLeod and Miles, 1988).

The differences could be due to species differences (*Quercus laevis* versus *Q. nigra*), methods of propagation or ontogenetic age differences, since initially pine plantlets were produced from cotyledonary explants while in this study they were produced from scapion materials of 3-year-

old trees. They were also different than those reported with *Eucalyptus viminalis* (Jarvis) (Bennett et al., 1964). They compared shoot growth of plantlets and seedlings on two different soil types. Seedlings accumulated more dry weight from 8 weeks through the 4 months in the greenhouse on lateritic soil, and no differences were observed on peat and sand soils. Here again, the differences may be due the propagation methods where they did not have elongation step. This may point to the possibility of carry-over effect from culture environment, or different species or soil potting mixture.

The relative distribution of dry matter between above- and below ground reflect genetic and environmental factors. A functional relationships was proposed to exist between root and shoots, where a balance was reached between roots and shoots (Sjost, 1971). Connell (1988) discussed that shoot:root ratio equilibrium tends to be adaptive. Auxinlike substances are used by shoots if photosynthesis is limiting and by roots if nutrients and water supply are limiting. Bloder and Beck (1988) also mentioned that availability of nutrients and moisture will result in higher shoot:root ratio. In this study seedlings had higher stem:root, leaf:root, and shoot:root ratio at each harvest (Table 1-4). Shoot:root ratio remained constant for the seedlings with values similar to that reported with five *Eucalyptus* species by Moseley et al. (1978), while plantlets

shoot:root ratio increased significantly at Harvest 2 and remained constant afterwards. However, when shoots were partitioned into stem and leaf, stem:root ratio increased with harvest and leaf:root ratio decreased with harvests for plantlets and seedlings (Table 3-5).

LEVINE (1983) indicated that developmental patterns which includes relatively greater allocation to roots during early growth stages could presumably indicate an adaptive advantage and tend to increase survival. The low above:below ground dry matter associated with plantlets may then be an adaptive mechanism to their new environment, since plantlets were transferred from their culture environment to a harsher new environment of the greenhouse. Another possible explanation is a carry-over effect from the IBA treatments at the rooting step. Regardless of the cause, the epirrhiza or the adventitious roots, especially at earlier stages, may be the reason behind the high survival percentage obtained in this study.

Seedlings showed higher net assimilation and relative growth rates at the early harvests which was associated with higher net photosynthesis,  $W_t$ , and chlorophyll content. This may reflect adaptive mechanism for seedlings. However, that decreased with harvest and the difference between seedling and plantlet relative growth rates decreased considerably by Harvest 5 (Table 3-22).

### Plant effects

Dry matter accumulation among plantlets showed no significant differences among parents coming out of the mist-greenhouse (Table 3-1). Differences became significant after Harvest 2 through 5 (Table 3-6 to 3-11). Plantlets from 1817 had more stem biomass and higher shoot:belowground ratios than plantlets from 1798. Similar results were observed with seedlings at Harvest 1, 3, and 4. At Harvest 2 and 4 although not significant, seedlings from 1817 had higher means while 1798 had lower means. This may point to genetic differences between these genetic lines and that genetic difference was expressed similarly by plantlets and seedlings. Genetic differences in dry matter allocation have been reported with crop plants (Evans, 1970), wheat and labially pine (Bultman, 1978). Zimmer and Brown (1988) correlated differences between ecotype species to habitat where seedlings from wet areas species showed higher shoot:root ratios. In this study, seedlings showed large variability among the three parents at the germinating stage (Table 3-1). Seedlings from 1817 had two times more stem and two times less root dry weights than 1798, indicating early expression of genetic differences.

### General Discussion

Plantlets have adventitious origin and were exposed to numerous factors during the propagation process. There

suspicious controls of development factors may lead to temporal or permanent physiological changes as compared to seedlings. In this study post-culture plantlet productivity was compared to their related field-grown seedlings at five harvests under stress greenhouse conditions. The data showed that plantlets differed from seedlings at early harvests (1 and 2) and the differences decreased or diminished with time. Ratios of plantlets to seedlings for all the parameters studied approached unity with time (Figure 3-4). Seedlings showed higher net photosynthetic rates, chlorophyll accumulation and nitrogen content and relative growth rate at Harvest 1 and 2. This may reflect the previous developmental control passed on to seedlings from the apical embryo. Plantlets, on the other hand, originated from adventitious organs may have had different physiological and genetical expression. However, the decrease in these differences indicates that plantlets adapted to their new environment similar to seedlings in a short time. By Harvest 3, plantlets were not significantly different from seedlings in most of the studied parameters. Plantlet shoot-growth to root-growth relative dry matter ratios continued to be significantly higher than seedlings. This could lead to higher survival and adaptability to field environment.

Differences among parents were only significant with dry matter accumulation in roots, shoots, and their ratios

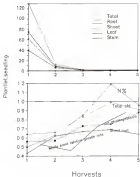


Figure 3-5. Plantlet:seedling ratios for stem, leaf, shoot, root, and total dry weight (upper) and net photosynthesis, total chlorophyll and foliar nitrogen percent (FNI), whole plant relative growth rate per day, and shoot:root ratio (lower) over harvests.

[Net photosynthesis and total chlorophyll content at Harvest 3 were predicted from regression models with foliar FNI].

among plantlets and seedlings. Parents showed similar patterns for plantlets and seedlings with those parameters. That may indicate that tissue culture conditions did not alter the genetic expression of plantlets.

These results could have significant practical application for the potential use of tissue culture for *E. grandis* in breeding programs and operational planting. It indicates that although differences occur, they last for only a short time, 10-20 days in the greenhouse. All that would need be plantlets produced by the methods described in Chapter 2 could show their genetic potential which was seen to be captured by direct micropropagation.

#### Conclusions

The main conclusion from this study is that plantlets differed from seedlings in the early stages of development, and that the differences decrease with time. Also plantlets showed similar trends to their related seedlings.

Net photosynthesis was found to be different only at Harvest 2, after 40 days in the greenhouse, and that was associated with high chlorophyll and foliar nitrogen content. Chlorophyll and nitrogen content per unit weight were also different at early harvests, and no difference was shown in the latter harvests.

Dry matter accumulation, relative distribution, and relative growth rates at the early stages of development

in the greenhouse were also different and these differences decreased with time. A similar trend was observed for the three genetic lines. Plantlets and seedlings at 1987 showed more above ground to below ground dry matter ratio while 1976 showed the opposite. This may point to the use of *E. grandis* plantlets for physiological and silvicultural studies.

This study was conducted to see whether plantlets are different from seedlings under normal greenhouse practices. Further research could screen for the causes of the differences observed in this study. It would be useful and interesting to study nitrogen fertilization effect on plantlets especially during the hardening step, and determine if it could result in higher nitrogen concentration and increased net photosynthesis rates immediately after the mist step. That way screen for nitrogen effect or simply age effect. Also monitoring of net photosynthesis just before and during the hardening stage will also shed some light to culture effect. Additionally, comparing plantlets from seedlings explants and from sprouts to their related seedling will also give information about age effect.

CHAPTER 4  
DIRECT REGENERATION OF L. GLABRA FROM  
TISSUE CULTURE EXPERIMENTS

Background

Somatic Embryogenesis

Somatic embryos, roots, and shoots *de novo* formation from plant tissue cultured *in vitro* is usually preceded by callus stage. Regeneration of plantlets from callus, usually referred to as indirect micropropagation, could be either through somatic embryogenesis or organogenesis. Somatic embryogenesis could have tremendous significance in forest tree breeding programs due to its potential of producing large numbers of plantlets from selected genotypes as well as its potential for generating variants. Somatic variation is the term used for the production and discovery of genetically (passive through seed generation) or clonally (can be maintained by continued cloning) variation. Such variations could result from the tissue culture process itself or processes of cellular changes in the original source material (Kawaroff et al., 1980; Hayles, 1983). Another potential use for somatic embryogenesis is for genetic engineering. Protoplasts from callus cells or suspension cultures are an input level for foreign DNA and fusion. Transfected protoplasts could be

induced to form colonies and then platelets through somatic embryogenesis to enter the breeding program.

The discovery of somatic embryogenesis is tied to the demonstration of totipotency of plant cells. It was first observed by Steward et al. (1958) in carrot. Since then carrot has become a model system and the source of much of the limited understanding of somatic embryogenesis.

Somatic (sexual) embryogenesis is the process of embryo initiation and development from cells that are not products of meiotic fusion (Tisserat et al., 1978). An embryo, a plant in the initial stage of development, is defined as a bipolar structure with root and shoot apical ends (Halperin and Githens, 1964). The morphology and developmental patterns of sexual and asexual embryos are very similar. They go through globular, heart and torpedo stages. Sharp et al. (1960) suggested two routes of somatic embryogenesis: 1) direct embryogenesis from tissues in the absence of cell proliferation, which occur through "pre-embryonic determined cells," where the cells are already committed to embryonic development and need only to be freed, and 2) indirect embryogenesis where cell proliferation is required. This occurs in differentiated, non-embryonic cells as "indirect embryogenic determined cells." The nature of the induction of embryonic cells or non embryogenic cells is completely unknown. Also the factors involved in regulation of

somatic embryos, once the cells are fastened towards maturation, are poorly understood (Geminato, 1961).

In spite of the fact that the understanding of factors and stimuli leading to somatic embryogenesis is fragmentary, it has been extensively observed in many plant species and families. This demonstrates it is a common phenomenon and the possibility exists that cells from any plant given the appropriate stimuli and conditions could be induced to form somatic embryos. It was observed with most systems that 1) the presence of an auxin or auxin-like substance, especially 2,4-D was critical for embryo initiation from single cells, and the lowering or complete removal of the auxin fosters maturation (stressed at all, 1947), and 2) reduced nitrogen was important for both initiation and maturation (Geminato and Stevard, 1971). The key variables in the embryogenic pathway include selection of the right explant, identification of embryogenic cultures, and formation of a defined media.

### The Explant

Embryonic, meristematic and reproductive tissues appear to have more potential for embryogenic growth. The most commonly used explants are from immature embryos, mature embryos, young inflorescences, young leaves, ovaries, shoot apices, and hypocotyle. For *Datura carota*, the most cultured plant, any part of the plant taken at any

time of development has successfully produced somatic embryos in culture. For some species, however, only certain tissues or organs respond in culture. Mammals in general respond when tissues of actively dividing cells are cultured. Somatic embryos have been used extensively as an explant in mammals. They were used in the quinea *Llama* (Kendrey et al., 1970) and *Llama* (Orie, 1960) and the mouse *Peromyscus* (Veall and Veall, 1961), *Peromyscus* (Lin and Veall, 1961). Embryogenic cultures in mammals have been derived from young vegetative material, such as basal part of young leaves (Kerns and Binkell, 1960) and somatic indifferentia (Binkell et al., 1960).

On the other hand, dicotyledonary plants showed more tissues and organs which respond to culture conditions. Production of *Carica papaya* (Clite and Gussner, 1960), ovules of *Glycine max* (Kocher and Spiegelberg, 1970), young leaves of *Gallia arabica* (Kordick and Sharp, 1977), hypocotyl of opium poppy (Sawler, 1961), leaf and shoot-tip of sweet potato (Lin and Ouchiffa, 1964), and cotyledons of walnut (Talschka and Moenchman, 1968).

### Identification of Embryogenic Cells

Identification of particular cells which give rise to embryos is usually difficult. Embryogenic cells for any species may have specific morphological, physiological, and cytological characteristics.

Morphological characters include color, degree of compactness, and texture. Tsaii and Tsaii (1983, 1984) described the embryogenic callus in germinase as white to pale yellow callus, often organized with a nodular and convoluted surface. Liu and Camilleri (1984) found embryogenic callus of sweet potato to be pale to bright-yellow, compact and organized callus. However, friable callus from maize was recently reported to be embryogenic. Gupang et al. (1985) described what they identify as embryogenic callus of *Eucalyptus "haichow"* as deep red, compact and superficially granulated.

The cytological features of the somatic embryo initials are: 1) rich in starch granules (Halperin 1978, Liu and Camilleri, 1984), and 2) small cells, relatively isodiametric, rich in cytoplasm, prominent of nuclei, thin-walled, and axially vacuolated (Sharp et al., 1988; 1979; Halperin, 1978).

## Media

Given an embryogenic tissue, development of somatic embryos occurs in a wide range of media depending on the species and the type of explant. All of the media used contain a balanced salt mixture, sugar, vitamins and, most importantly, growth regulators. Weiss et al. (1981) reported that 70% of embryogenic calli were cultured on Murashige and Skoog media (MS) (1961).

A key element of the M medium is the presence of high levels of nitrogen in the form of ammonium and nitrate. The source of reduced nitrogen can also be in the form of complex addenda such as coconut milk, or single amino acid such as L-glutamine (Kusuda and Kusuda, 1970) or L-alanine (Wetherall and Inquill, 1974). The ammonium ion can in itself serve as a sole source of nitrogen (Aminato and Sheward, 1971).

Of the other salts present in all culture media, potassium is essential for somatic embryogenesis (Bouart et al., 1967). Iron was also reported to be important and effective in embryo development from the globular stage (Aminato, 1967). It is present in M as a chelated form.

The growth regulator auxins in combination with cytokinins appear to be the most critical and essential factors for the onset of growth and induction of embryogenesis (Fajlura and Rossmann, 1969). Some species like carrot readily produce an embryogenic culture with a range of auxins (Aminato, 1967). But 2,4-D at 0.05-0.5 mg/l was the most effective and widely used along with kinetin. Higher levels of 2,4-D have been reported for *Rhonda dentilifera* (Depoide and Harschke, 1970). Maturation of embryos, however, requires removal or reduction of 2,4-D concentration, on secondary medium.

Carbohydrates are also important in the process of embryogenic induction. Sucrose in particular, appears to

be the most effective reduced carbon source. However, sucrose, galactose, and lactose at ranges of 18 to 48 were reported to enhance embryo formation (Giles-Alex and Veal, 1988). Gungor et al. (1991) reported a concentration of 4.5-5.8% to be effective for morphogenic callus of eucalypts.

It is clear that there are many variables in the nutrient media which can influence the embryogenic development. However, most of them do have an enhancement effect and the main critical requirement is the actual concentration of auxins in the primary and secondary media.

#### ***Eucalyptus* Indirect Micropropagation**

Callus cultures have been established from a number of *Eucalyptus* species explants from both juvenile and adult plant materials (Burnard-Cresswell et al., 1983; Moench and Bennett, 1984). The most used hormones were the auxins IAA, NAA, and IBA alone, or in combination with the cytokinin media or BAP with large variation in the hormonal levels which have induced callus (Kitahara and Oikawa, 1979; Bennett and Moench, 1982; Burnard-Cresswell et al., 1983; Bennett and Moench, 1984).

Regeneration from callus culture has been reported in few studies and mostly from seedling explants. These included hypocotyl of *E. alba* (Kitahara and Oikawa, 1979), seedling hypocotyls and cotyledons of *E. chlorodonta* (Kawaja

and Atai. Inoue (Lakshai site 1978, 1980), whole seeds or seedling pieces of *E. blainovii* (Qupang et al., 1981), cotyledons and stems of *E. grandis* (Bennett and McCook, 1981), seedling shoots, roots, hypocotyle and cotyledons of *E. ammannioides* (Mehre-Palita, 1982), and hypocotyle of *Eacalyptus glaberrima* (Ota et al., 1982). In all of these reports, explants were from juvenile seedlings except where stem elements of *E. grandis* were used (Bennett and McCook, 1982). Regeneration was mostly through organogenesis and formation of shoots. However, embryoid development of *E. grandis* without regeneration was claimed by Lakshai site (1984), and somatic embryogenesis was claimed by Qupang et al. (1981) with *E. blainovii* from nodules and callus. No anatomical or histological work was reported with these reports.

*E. grandis* was found difficult to regenerate from callus. On Forward (1978) mixed *E. grandis* callus with tobacco hoping that a diffusible factor from the tobacco might induce it to regenerate. No regeneration was observed, however, massive roots were observed.

### Objectives

The main goal of this study was to develop a regeneration system from callus through somatic embryogenesis for this important species. The specific objective was to test the hypothesis that *E. grandis* has

embryogenic totipotency and can be induced to express that potential. In order to test this hypothesis, three subhypotheses were tested:

- 1) Embryogenic development from somatic cells depends on the kind of tissue used as explant. In other words, *E. grandis* possesses certain tissues and organs which are more responsive than others.
- 2) Embryogenic callus is distinctly different from other types of calli. It is characterized by special morphological and cytological features. Moreover, cells of embryogenic callus divide in a distinct manner to form glabrous, heart and torpedo stages.
- 3) Well defined nutrient media are required for induction of embryogenic pathway and maturation of somatic embryo.

#### Material and Methods

*E. grandis* hybrid callus was induced from different explants on primary media with a combination of a wide range of auxin and cytokinin concentrations. Calli were then transferred to secondary media with lower concentration of auxin and cytokinin to attempt to induce somatic embryo development and maturation. In the process, embryogenic callus and what type of explant was more responsive to culture conditions were assessed. Unless mentioned otherwise, certain conditions were used in all

the experiments. The basal nutrient medium consisted of Murashige and Skoog (1962) salts with Gamborg et al. (1968) (B5) vitamins, supplemented with 25 g/l sucrose, 100 mg/l glutamine, 10% coconut milk and 1.0 g/l of agar. Media pH was adjusted to 5.8 before autoclaving. Plant material and media were sterilized as generally described in chapter 1. Cultures were initiated in the primary media under dark conditions with temperature of 28° C and 22° C day and night respectively. Cultures on secondary media were kept under 12 hours of day and night with light intensity of average photon flux density of photosynthetically active radiation (PAR) of 10  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

### The Explants

The following were used as explants: immature inflorescences, floral parts (anther filaments and ovary tissues), anthers, hypocotyle of young seedlings, shoot tips from 2-month-old sprouts and shoot tips from sprouts subcultured several times in multiplication medium as described in chapter 1. Explants were collected from 5-year-old trees (same clones used in Chapters 2 and 3 (clones 2813, 2814, and 2888 at study ORSL-37 at Fakahia, Florida)). Immature inflorescences were collected and experimented with in July 1981 and 1987. Anther filaments and ovaries were excised from floral buds collected in August 1987. Shoots from sprouts (from trees girdled in

February 1987) were collected in May 1987. Open-pollinated seeds were also collected from the same clones to provide somatic embryo explants and seedlings for hypocotyl explants.

## Media

Initial studies showed that callus can be induced from immature inflorescences on up to 18 mg/l of 2,4-D. Accordingly concentration of 2,4-D in the primary media was tested in a factorial of seven levels of 2,4-D (1, 2, 3, 4, 6, 8, 10 mg/l), and two levels of kinetin (0.0, 1.0 mg/l) (Table 4-1). Calli were then transferred to secondary media after 3-4 weeks in the primary media. Secondary media tested contained reduced hormone concentrations of 1/4 and 1/2 and 0.5 of that in the primary media. Sucrose was also reduced to 30 mg/l.

Failure to obtain somatic embryogenesis with 2,4-D led to experimentation with BA at 0, 4, 3, 1 mg/l in combination with 1, .5, and 0.5 mg/l of kinetin in the primary media. Secondary media also contained reduced hormone concentrations to 1/4, 1/8 and 0.5 of that in primary media and 30 mg/l of sucrose.

## Design and Statistical Analysis

In the primary media tested, 10 explants per type distributed over four plates were cultured per treatment.

Callus mass and compactness and root formation were scored in a 1-4 scale. Light microscopy was performed regularly to observe organization of callus and formation of somatic embryos, shoots, and roots. The callus from each initial explant in the primary media was divided into three and subcultured into each of the secondary media tested. Also regular light microscopy was done regularly to observe if there was formation of somatic embryos at any stage of development (globular, heart, and torpedo) and organogenesis by formation of either shoots or roots.

K-S (1964) procedure SPSSIMAT was used for the analysis of callus mass and compactness data. The Kruskal-Wallis, the nonparametric one-way analysis of variance by ranks, option was used to test the same with the test statistic  $H$  as

$$H = \frac{12}{n(n+1)} \left\{ \sum_{j=1}^K \frac{n_j^3}{n_j} \right\} - 3\{n+1\}$$

where

- $K$  = number of groups.
- $n_j$  = number of observations in the  $j$ th group.
- $n$  = number of observations in all groups combined.
- $R_j$  = sum of the ranks in the  $j$ th group.

### Callus

Somatic embryos were not observed under the experimental conditions used in this study. Induction of callus on the primary media was achieved with all of the explants used. Callus amounts and morphology depended on the kind and concentration of hormones and the type of explants. Use of 2,4-D in the primary and secondary media induced callus in concentrations up to 10 mg/l for certain types of explants, while shoot and juvenile explants did not tolerate as high a concentration of 2,4-D. Use of B6A in the primary media produced morphogenic callus on hypocotyle from one-week-old seedlings.

### Response of Explants on 2,4-D Media

Immature inflorescences produced different types of calli, usually at the cut end of the peduncles, with different colors, compactness, and callus mass on the primary media (Table 4-1, Figure 4-1A). Callus color ranged between whitish, yellow, and greenish yellow. Compactness of callus increased significantly with decreasing 2,4-D and also with the presence of kinetin at 1 mg/l in the primary media. Callus was produced in up to 10 mg/l of 2,4-D with or without kinetin, with higher mass at 1 mg/l of 2,4-D and no kinetin. No organization or any sort of differentiation of root, shoots, or somatic embryos were observed. Transfer of calli from primary media to secondary media was usually

Table 4-3. *E. grandis* sapling color, size, compactness and root development from different types of explants at seven concentrations of 2,4-D and two concentrations of kinetin (K) after four weeks.

| Explant type,<br>2,4-D $\mu$<br>mg/l | Color | Mass <sup>1</sup> Compact. <sup>1</sup> | Roots <sup>2</sup>              |
|--------------------------------------|-------|-----------------------------------------|---------------------------------|
| Immature inflorescences              |       |                                         |                                 |
| 1.0                                  | 0.0   | White & Yellow                          | 3 <sup>a</sup> 3 <sup>a</sup> 0 |
| 0.5                                  | 0.0   | White & Yellow                          | 3 4 0                           |
| 3.0                                  | 0.0   | Yellow                                  | 3 3 0                           |
| 4.0                                  | 0.0   | Yellow                                  | 3 3 0                           |
| 0.5                                  | 0.0   | Pale Yellow                             | 3 3 0                           |
| 0.5                                  | 0.0   | Pale Yellow                             | 3 4 0                           |
| 10.0                                 | 0.0   | Pale Yellow                             | 1 1 0                           |
| 1.0                                  | 1.0   | Yellow                                  | 3 3 0                           |
| 3.0                                  | 1.0   | Yellow                                  | 3 3 0                           |
| 3.0                                  | 1.0   | Orange Yellow                           | 3 3 0                           |
| 4.0                                  | 1.0   | Orange Yellow                           | 3 4 0                           |
| 0.5                                  | 1.0   | Orange Yellow                           | 3 3 0                           |
| 0.5                                  | 1.0   | Orange Yellow                           | 1 3 0                           |
| 10.0                                 | 1.0   | Orange Yellow                           | 1 3 0                           |
| Coppice shoot tips                   |       |                                         |                                 |
| 1.0                                  | 0.0   | Pale Yellow                             | 3 <sup>a</sup> 4 <sup>a</sup> 1 |
| 3.0                                  | 0.0   | Pale Yellow                             | 4 3 1                           |
| 3.0                                  | 0.0   | Yellow                                  | 3 3 0                           |
| 4.0                                  | 0.0   | Yellow                                  | 3 3 0                           |
| 0.5                                  | 0.0   | Yellow                                  | 1 1 0                           |
| 0.5                                  | 0.0   | 0                                       | 0 0 0                           |
| 10.0                                 | 0.0   | 0                                       | 0 0 0                           |
| 1.0                                  | 1.0   | Pale Yellow                             | 3 3 0                           |
| 3.0                                  | 1.0   | Pale Yellow                             | 4 4 0                           |
| 3.0                                  | 1.0   | Yellow                                  | 3 4 0                           |
| 0.5                                  | 1.0   | yellow                                  | 3 3 0                           |
| 0.5                                  | 1.0   | yellow                                  | 1 3 0                           |
| 0.5                                  | 1.0   | 0                                       | 0 0 0                           |
| 10.0                                 | 1.0   | 0                                       | 0 0 0                           |

/ Ranked in a scale of 1-4 with 1 indicates least and 4 max.  
<sup>a</sup>0<sup>a</sup> indicates no analysis was observed.

\* Indicates significant variation between treatment for that specific explant with Student-Welch's nonparametric test.



Figure 4-5. *E. granata* callus on primary media; a) at the petiole end of lettuce inflorescence on 2 mg/l of 2,4-D, and b) morphogenic hypocotyl hairy red pigmented callus on 4 mg/l of BSA and 2 mg/l of kinetin.

accompanied with browning and eventual death of the calli cells.

Stem filaments from floral buds produced very friable and soft callus, as compared to the immature inflorescences, at 1 and 2 mg/l of 2,4-D with 1 mg/l kinetin. Higher 2,4-D concentration resulted in browning and death of the explants. The soft friable callus of the stem filaments was also non-suspensoid when transferred to secondary media. Culture of ovaries produced large amounts of phenolic, presumably due to injuries from the extraction procedures, and they eventually died.

Callus was also produced on shoot tips cultured directly from coppice material and from coppice material subcultured in multiplication media (as described in Chapter 2) in concentrations up to 2 mg/l of 2,4-D (Table 1). Higher concentrations tended to kill the shoot tip or produce a small brown mass of callus. Similar to immature inflorescences, compactness of the callus increased with decreasing 2,4-D concentration and with the presence of kinetin. Continuous root production was observed on 1-2 mg/l of 2,4-D with no kinetin. Transfer of callus from the shoot tips resulted in browning and death of the callus cells similar to that of floral parts.

Gynodio embryos and gynodioyl from one-year-old seedlings showed similar response to that of stems with 2,4-D, but more roots were observed and the callus was more

nodular, and usually whitish yellow and occasionally red pigmented. Transfer of such callus to secondary media resulted here again in browning and death of callus.

#### Response of Explants to BAA

BAA treatments produced less variable callus than 2,4-D treatments for most of the explants. Immature inflorescences produced callus at concentrations higher than 2 mg/l of BAA with no significant difference between the treatments in terms of callus compactness, mass, and color. Callus tended to be nodular and creamy yellow in color. No differentiation of embryos, shoots, or roots was observed on the primary or secondary media. Use of subcultured shoots produced similar results, with occasional root formation on media free of kinetin.

Hypocotyl cultures, on the other hand, produced shiny red pigmented callus on 4 mg/l of BAA and 1 mg/l of kinetin (Figure 4-18). A degree of organization accompanied with root formation were observed after 4 weeks in culture. Transfer of such callus to a hormone free medium produced highly morphogenic nodular thallus, with massive number of shoots and shoots primordia (Figure 4-19). Thallus were successfully attached to grow in a medium with low auxin and cytokinin concentration, similar to that described by Guyot (1964). Rooting was also achieved on low hormonal concentration of IBA (.1-.3 mg/l).

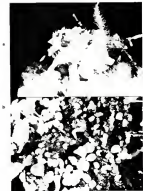


Figure 4-5. *E. grandis* callus differentiation on hormone from secondary media: a) after one week, b) formation of shoots after 4 weeks.

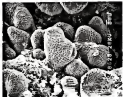
The nodular tissue masses of four genetic lines have been sub-cultured for over ten years on 1/4 MS now with the same regenerative ability when transferred to shoot enhancement media.

### Discussion

A key element in the utilization of the full potential of tissue culture for eucalypts, and woody perennials in general, is the development of regeneration systems from callus. In this study, unlike the hypothesis, regeneration through somatic embryogenesis was not possible under the experimental conditions used. However, highly morphogenic callus from hypocotyl explants was induced which produced massive numbers of shoot primordia (Figure 4-1) and adventitious shoots through organogenesis (Figure 4-2, 4-3). Such callus is similar to that reported by Quyang et al. (1981).

### Somatic Embryogenesis

Facilitating somatic embryogenesis was reported by Labalai Sita (1984) and Quyang et al. (1981), where they did not definitely identify somatic embryos. Labalai Sita (1984) described formation of embryo-like structures, which did not develop into plantlets and no histological work was reported. In the other study, Quyang (1981) reported similar callus to the one observed in this study. However,



Figures 4-5. *E. grandis* scanning micrographs of shoot primordia on hormone free secondary medium.

The morphogenesis observed in that study seems more like organogenesis than somatic embryogenesis, since they had to induce shoots and roots separately with hormonal treatments on what they mentioned as somatic embryos. In this study a large combination of hormonal treatments with different types of explants was tried to induce embryogenic pathway. Under the conditions used embryos or embryo-like structures were not observed. This could be tied to the complexity and the precise developmental processes associated with somatic embryogenesis. It is possible that some or all of the experimental conditions followed in this study were not the appropriate stimuli which could induce the embryogenic development pathway of *E. grandis* hybrids.

The key factors which appear to control or enhance the somatic embryogenesis process include the kind, concentration, and balance of hormones in the media, the explant, and identification of the embryogenic culture (Assineto, 1981; Yeeli and Yeeli, 1984). The kind, balance and concentration of growth regulators in the primary and secondary media in addition to the basal media have a large effect on the embryogenic induction (Assineto, 1983). This study used 2,4-D (the mostly used auxin for the induction of embryogenic culture (Assineto, 1983)) and 6BA with kinetin in the primary media and reduced concentration in the secondary media. Induction of callus was usually possible on 2,4-D primary media with most explants (Talia

4-1). However, organiation and differentiation of the callus were very limited, with usually no differentiation of shoots or embryos. This may indicate that 2,4-D under the experimental conditions described in this study is not the appropriate hormone for *E. granata* embryogenic callus induction. However, it is also possible that other factors rather than 2,4-D itself were the reason behind the observed response. These factors include the type and concentration of the other growth regulators, the explant, the basal nutrient media, and the physical conditions around the culture. The explant age, type and origin, and the physiological state of the shoot plants have a large impact on embryogenesis (Thorp, 1981). Although this study investigated some of the commonly used organs and tissues for somatic embryogenesis, they may not be the most responsive tissues for *E. granata*, or they were not at their optimal developmental stage.

Another key factor is the early identification of the embryogenic callus. Weill and Weill (1984) emphasized the importance of early identification, selection and culture of embryogenic callus developed in the primary media. They mentioned that each callus is usually associated with other type of calli which grow fast and can usually overgrow the embryogenic callus (Weill and Weill, 1984). It's very difficult sometimes to identify each callus, unless there is prior knowledge about its morphological and histological

features. So there is a possibility that embryogenesis medium was induced, but was not identified and was overgrown by other calli.

Accordingly, no conclusive judgment is made that these *E. grandis* hybrids do not have the potential to form somatic embryos, especially, since somatic embryogenesis has been successfully observed in many other plant species and families. Further experimentation with growth regulators, basal media, explants, and physical environment is needed to precisely identify the appropriate features and stimuli for *E. grandis*.

#### organogenesis

Although somatic embryogenesis was not achieved, organogenesis was observed on BSA primary explants rather than 1,4-B- BSA and kinetins induced more organized callus with root differentiation from shoot tips and syzytic embryos and highly morphogenic callus from hypocotyl explants (Figure 4-1b). Eucalyptus hypocotyls were reported in other studies to be more responsive to culture than other type of explants (Hibshara and Cohen, 1979; Knejs and Stal, 1980; Lakshmi Sita 1979, 1981; Mehra-Raiz, 1981; Oka et al., 1982; Qinyang et al., 1981).

The morphological characterisation of the hypocotyl morphogenic callus obtained in this study on 4 mg/l of BSA and 1 mg/l of kinetin confirmed the Hibshara group's (Qinyang

et al. 1981) red pigmented nodular callus as more morphogenic than other types of calli. Other red nodular pigmented callus from hypocotyle was reported by Ishihara *et al.* (1978) and Shi *et al.* (1982), where limited regeneration of plantlets through organogenesis was obtained. In this study, however, transfer of such callus to media with low hormonal concentration produced nodular tissues and roots and then large number of shoot primordia within four weeks (Figures 4-2, 4-3). Similar morphogenic response was reported by Qiyang *et al.* (1981), which they described as somatic embryogenesis development. The high shoot organogenesis observed in this study and Qiyang (1981) compared to the other *Eucalyptus* reports may be related to the reduction of the hormonal concentration in the secondary media. In all the other reports with *Eucalyptus*, morphogenic callus was transferred to media with relatively high cytokinin concentration (1.5 mg/l or more).

In order to maintain the nodular tissues and shoot primordia and suppress further shoot development, reduced basal media was found useful. Maintenance of the nodular tissues and shoot primordia was achieved on a simple hormone free medium which contains 1/4 MS, 10 mg/l of sucrose, and 3.5% coconut water on a monthly transfer schedule. It has been subcultured so far for 2 years without losing its regenerative power. This would have

significant practical applications including a ready source of regenerative material and conservation of specific genotypes. Loss of regeneration abilities with subculture was reported with some species (Bonga, 1980).

For general vegetative propagation, the organogenesis observed with the hypocotyl explants can provide large numbers of plantlets. However, that may not be optimal for genetic engineering purposes. Somaclonal variation can be possible through this organogenesis, however, there is chance for chimeric tissues since plantlets can not be traced back to single cell origin as would be the case with somatic embryogenesis.

### Conclusions

Somatic embryogenesis of *E. canadensis* hybrids was not possible under the experimental conditions used in this study. Explant response to 2,4-D varied with tissues and organs. Floral buds were more tolerant to 2,4-D than other types of explants, producing healthy callus in up to 10 mg/l of 2,4-D. Also callus color, compactness, and quantity depended on the explant origin.

Floral tissues appeared to be more responsive to hormonal treatments than other type of explants. Hypocotyle from germinating seedlings produced highly embryogenic callus on 4 mg/l of NAA and 1 mg/l of Kinetin under dark conditions. The morphological characteristics of such

callus confirmed other reports that *Eucalyptus* morphogenic callus is generally nodular, compact, and red pigmented. The morphogenic response of each callus when transferred to media with reduced hormonal concentrations deserves further study. The impressive differentiation of shoots and shoot primordia may indicate totipotency or some genetic control of auxilipines to produce shoots. Further study of this phenomenon may lead to understanding of coppicing, the natural de novo development of shoots associated with cutting or girdling of *Eucalyptus* stems.

Shoot tips from sprouts subcultured several times on multiplication media (Chapter 3) produced nodular and pigmented callus on NAA primary media, but failed to regenerate when transferred to secondary media. Such callus holds the promise for regeneration. In this type of explant lies the most potential for indirect regeneration of genetically field-tested *Eucalyptus* lines. Further subculture of shoot on cytokinin multiplication media may lead to rejuvenation of shoots to the point where it can respond to culture and produce more morphogenic callus.

Further studies to induce somatic embryogenesis can be directed to further trial with growth regulators, especially NAA, and IAA. Also, some hypocotyls and juvenile material seem to be more responsive to culture. Their use first as explants to identify embryogenic callus characteristics should precede use of mature tissues.

## CHAPTER 5 GENERAL CONCLUSIONS

Vegetative propagation of eucalypts is a useful tool for immediate capturing of genetic potential of elite trees. Cloning through rooted cuttings was successful with large genetic gains reported in Australia, Brazil. Tissue culture cloning could serve as a better alternative with tremendous potential for tree improvement programs, afforestation, physiological and silvicultural studies where genetic uniformity is desired, and for conservation of germplasm. To fully realize and use this potential, for any specific purpose, a suitable regeneration system has to be developed. To be biologically and economically sound such a system has to consider the quality and quantity of the regenerates. This study was conducted to develop direct and indirect regeneration systems and shed light on these two major aspects for selected *Eucalyptus grandis* hybrids.

Direct micropropagation was successfully made that a million plantlets for operational planting can be produced from a single rooted segment. The key factors for the success of this method were the forcing of relatively juvenile sprouts from mature trees by girdling and their use as explants. Also, continued subculturing in a cytokinin medium contributed significantly to increasing

the rooting success (which is considered as one of the major problems of analytic propagation).

Further research is needed to reduce the cost by developing automated systems. The uniformity and the clump subculture of shoots can aid in developing such a system. Direct rooting of clumped shoots in mist-greenhouses would eliminate the in vitro rooting step. Research efforts can also concentrate on understanding the juvenility aspects observed with apical and axillary subculturing in cytokinin medium. This could add to the understanding of physiological mechanisms controlling these natural phenomena. Epiphyseal studies with relation to development of strong shoot is another important aspect which should be considered for further research.

Directly micropropagated plantlets of *E. grandis* hybrids showed high survival rate with no sign of abnormal physiological or growth behavior in the greenhouse. Compared to seedlings, plantlets differed mostly during the early post-culture development in all the studied parameters, and the differences decreased with time. The five month greenhouse study showed that seedlings had higher net photosynthesis rates only after the first 40 days in the greenhouse following the mist phase. That was explained by higher efficiency of the young seedlings. However, after 80 days in the greenhouse seedling net photosynthesis dropped to that of plantlets. Similar

results were observed with chlorophyll and nitrogen concentrations. Plantlet and seedling percent responses over time may reflect no change in plantlet physiological responses to changing environmental conditions.

Plantlet dry matter accumulation and distribution was also significantly different from seedlings, and the differences decreased with time. Plantlets exhibited greater root growth during their early development and lower relative growth and net assimilation rates. The similar ranking of the parents among plantlets and seedlings for shoot and root dry weights and shoot:root ratios may indicate genetic control over these traits that were expressed similarly by plantlets and seedlings.

These results suggest that under practical production conditions, deleterious physiological differences which could influence future performance of *Eucalyptus nitens* hybrid seedlings and plantlets are not apparent. This suggests that culture conditions did not alter the genetic potential of plantlets. An ongoing field experiment showed 100% plantlet survival and no significant differences in 5-month and 7-month height measurements of plantlets and seedlings. These results clearly demonstrate that tissue culture derived plantlets have the potential to capture genetic gains of selected *E. nitens* hybrid trees, and recommend the use of tissue culture as an integral part of tree improvement programs. Further research is needed to

understand if the differences between seedlings and plantlets at the early stage of development are due to culture conditions or physiological age.

Indirect micropropagation attempts from mature-tree-explants were not successful and have enhanced the importance of juvenility. Juvenile explants from one-week-old seedlings were more responsive to hormone treatments and produced highly morphogenic callus, which differentiate into massive number of shoots and roots. The morphological characterization of such callus confirmed other reports that *Eucalyptus* morphogenic callus is generally nodular, compact, and red pigmented.

Somatic embryogenesis was not possible under the conditions described in chapter 4. Further research on this area is needed. The use of embryogenic explants can add in developing the spaten factor and then it can be applied to other types of explants. Further studies can also concentrate on the depressive shoot and root differentiation observed.

APPENDIX 1  
 SEPARATION AND GROWTH (1967) (PH) BASAL MEDIUM

| Ingredient                                         | Amount (mg/l) |
|----------------------------------------------------|---------------|
| <b>Macrominerals</b>                               |               |
| MgSO <sub>4</sub> 7H <sub>2</sub> O                | 370           |
| KCl, 6H <sub>2</sub> O                             | 170           |
| NaCl                                               | 1800          |
| Na <sub>2</sub> SO <sub>4</sub>                    | 1850          |
| CaCl <sub>2</sub> 2H <sub>2</sub> O                | 480           |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>    | 50            |
| <b>Microelements</b>                               |               |
| B <sub>2</sub> HPO <sub>4</sub>                    | 0.1           |
| Na <sub>2</sub> MoO <sub>4</sub> 2H <sub>2</sub> O | 15.4          |
| NaNO <sub>3</sub> 7H <sub>2</sub> O                | 0.4           |
| Na <sub>2</sub> SeO <sub>3</sub> 2H <sub>2</sub> O | 0.15          |
| CuSO <sub>4</sub> 5H <sub>2</sub> O                | 0.005         |
| CoCl <sub>2</sub> (6H <sub>2</sub> O)              | 0.005         |
| KI                                                 | 0.005         |
| FeSO <sub>4</sub> 7H <sub>2</sub> O                | 0.005         |
| Na <sub>2</sub> EDTA                               | 0.005         |
| <b>Lactose</b>                                     | <b>30,000</b> |
| <b>Vitamins</b>                                    |               |
| Thiamine HCl                                       | 0.1           |
| Pyridoxine HCl                                     | 0.1           |
| Biotin acid                                        | 0.5           |
| Nyo-Inositol                                       | 100           |

pH adjusted to 5.6.

APPENDIX 3  
MODEL AND ANALYSIS OF VARIANCE FOR MULTIPLICATION  
AND ELONGATION TREATMENTS

$$\text{Model } Y_{ijk} = \mu + C_i + T_j + (CT)_{ij} + E_{ijk}$$

where

Y = Response ( Multiplication or Elongation)  
 $\mu$  = mean  
 C = Clone effect (fixed)  
 T = Treatment effect (fixed)  
 CT = Clone X Treatment interaction effect  
 E = error.

ANOVA table for multiplication and elongation

| SOURCE              | DF | Mean square | F Value | Prob F |
|---------------------|----|-------------|---------|--------|
| 1) Multiplication   |    |             |         |        |
| Clones              | 3  | 69.23       | 0.88    | 0.5095 |
| Treatments          | 5  | 78435.648   | 100.22  | 0.0001 |
| Clones x Treatments | 15 | 328.85      | 0.51    | 0.9768 |
| Error               |    | 217.57      |         |        |
| 2) Elongation       |    |             |         |        |
| Clones              | 3  | 52.84       | 1.48    | 0.3389 |
| Treatments          | 5  | 4899.93     | 113.84  | 0.0001 |
| Clones x treatments | 15 | 18.18       | 0.52    | 0.9841 |
| Error               |    | 35.28       |         |        |

APPENDIX 3  
CRIBBWORK DESIGN FOR THE COMPARATIVE STUDY OF  
PLANTLETS AND SEEDLINGS.

| Block 1 |   | Block 1 |   |
|---------|---|---------|---|
| 1       | 4 | 4       | 1 |
| <hr/>   |   |         |   |
| 3       | 2 | 2       | 3 |
| <hr/>   |   |         |   |
| Block 1 |   | Block 1 |   |
| 2       | 3 | 4       | 1 |
| <hr/>   |   |         |   |
| 4       | 1 | 3       | 2 |
| <hr/>   |   |         |   |

Each block was divided into 4 plots. Plots 1, 2, 3, and 4 were harvested on May 9, 1968 (Harvest 1), June 18, 1968 (Harvest 2), July 28, 1968 (Harvest 3) and September 9, 1968 (Harvest 4) respectively.  
(16 seedlings and 4-6 plantlets per genetic line were randomly distributed within each plot).

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

  
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